CHRONIC EFFECTS OF ADVERSE WATER QUALITY ON THE GREENLIP ABALONE, *Haliotis laevigata* DONOVAN

by

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ABSTRACT

Bioassays were undertaken to assess the effects of ammonia, nitrite, dissolved oxygen and pH on greenlip abalone, *Haliotis laevigata*. At the end of each chronic bioassay, oxygen consumption, haemolymph ionic concentration and tissue microstructure were documented, relative to toxicant concentration.

EC_5 data (concentration causing a 5% growth reduction; whole wet body weight unless stated) indicated that these abalone were very sensitive to elevated levels of ammonia (0.041 mg FAN.l^{-1}), low dissolved oxygen (7.36 mg DO.l^{-1}) and low (7.78) and high (8.77) pH. Most of these variables affected shell growth relative to whole body growth, indicating some independence of nett shell and soft tissue growth rates. Greenlip abalone were also sensitive to nitrite on a growth basis. Modeling of the whole weight data indicated relatively uniform growth depression regardless of concentration in the range 0.56-7.80 mg NO_2-N.l^{-1}.

The influence of nutritional history on the susceptibility of abalone to ammonia was determined in an acute bioassay. The abalone had been maintained on either a mixture of three commercial diets, or the same mixture treated at 110°C for two days. No significant difference in mortality occurred between the two diet groups (p>0.05). At 1.025 mg FAN.l^{-1}, an LT50 value of 125.3 h was estimated by probit analysis.

Oxygen consumption patterns were similar to growth trends (depressed consumption per unit whole weight per unit time in slow growing groups) for nitrite, dissolved oxygen and pH. However, oxygen consumption was elevated at higher ammonia concentrations.

In general, tissue histology was a relatively insensitive indicator of growth rate inhibition as structural changes were usually only pronounced at extreme concentrations. Gill structure was affected by exposure to high nitrite and low dissolved oxygen levels, with ciliates occurring between the gill lamellae of abalone exposed to low dissolved oxygen. Kidney tissue exhibited changes from exposure to ammonia and nitrite.

Haemolymph ionic patterns did not provide any apparent stress specific indicators of growth depression. However, reduced haemolymph sodium and chloride concentrations were found in abalone exposed to ammonia, and nitrite respectively.

In most chronic bioassays, control groups exposed to saturated seawater survived well (>95%). Growth rates were progressively improved throughout the series of bioassays by adding heaters and later, submersible pumps to increase current flow. However, growth rates were depressed in the absence of within-tank aeration in the dissolved oxygen bioassay.

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ABBREVIATIONS

DO	Dissolved oxygen
TAN	Total ammonia nitrogen (NH_3-N plus NH_4^+-N)
FAN	Free (unionised) ammonia nitrogen (NH_3-N)
NO_2-N	Nitrite nitrogen
SGR	Specific growth rate (%.day^{-1}); $\frac{[ln(Final) - ln(Initial)] \times 100}{Days}$
WWBW	Whole wet body weight
SL	Shell length
EC_x	Estimated concentration where growth is reduced by x %
BOD	Biological oxygen demand
LT50	Median lethal time
SE	Standard error
All values given as mean±SE, unless otherwise stated	

1 GENERAL INTRODUCTION

The value of the Australian abalone export industry in 1994 was $AUS 33 million (Anon 1997). Included in this value are the figures for the preliminary production of cultured abalone, expected by commercial aquaculturalists to total up to 800 tonnes per annum by 2001 (Maguire and Hone 1997). The decline in world production in the past few decades (Rudd 1994) is now reflected in the increasing demand on aquaculture to meet the worldwide demand for abalone (Fleming and Hone 1996).

Initial investigations into abalone culture in Japan led to major aquaculture production (Ino 1951). Despite attempts to use the technology devised for Japanese abalone on Australian species (Cropp 1989), it was not until progress was made in artificial diet research that culture of Australian abalone made advances (see Hone and Fleming 1996, Hone 1996, 1997, 1998). With these increases came the need to understand the biology of Australian abalone, as past experience has shown that many aspects of abalone culture must be tailored to suit the local species and environmental conditions (Fleming and Hone 1996). A more complete understanding of the interrelationships between aquatic productivity and water quality is essential to ensure the continued growth of an aquaculture industry (Tomasso and Brune 1991), such as abalone farming.

The two major species in the Australian abalone fishery, the greenlip abalone, *Haliotis laevigata* Donovan and blacklip abalone, *Haliotis rubra* Leach, have also been the priority species for aquaculture in Australia (Maguire and Hone 1997). Wild greenlip abalone, *Haliotis laevigata*, occur on either low, sloping rocks or at the base of steep gutters and clefts from 10-25 m depth, although in calmer water they inhabit shallower waters (Shepherd 1975). Blacklip abalone, *Haliotis rubra*, prefer a more cryptic habitat, though are also found on open, vertical rock faces (Shepherd 1975). Both greenlip and blacklip abalone are found in Western Australia, South Australia, Victoria and Tasmania, with blacklip abalone also being found in New South Wales. Postlarval abalone settle most commonly on coralline algae (Morse and Morse 1984) and remain there until 10 mm in size, when they move to cryptic sites under boulders (Shepherd 1973). Both these species can reach 200 mm in length (Shepherd 1973) and have similar temperature preferences of 18.3 and 17.0 for juvenile greenlip and

blacklip abalone, respectively (Gilroy and Edwards 1998). Greenlip abalone are known to have a preference for some degree of shading or refuge cover (Maguire et al. 1996a), and, if appropriately fed, are known to tolerate 28 ppt salinity for 96 hours, although survival was reduced at 23 ppt salinity (Boarder 1997). Growth rates of abalone in the wild have been noted at up to 1.69 mm per month for greenlip abalone for the first five years, with declining growth rates after this (Shepherd 1988). Greenlip abalone held in experimental systems have achieved growth rates in excess of 5 mm per month (Higham et al. 1998). In the wild, greenlip abalone have a preference for drift algae, most commonly Rhodophytaceae species (Shepherd 1973). Current investigations into abalone nutrition have produced successful artificial diets (Hone and Maguire 1996).

Observations on behaviour of animals in wild conditions are the most likely source of the preliminary biological requirements of an aquaculture species. Shepherd's (1973) study on the ecology of several Australian abalone species suggested several factors that could affect the culture of these species. Probably the most notable example has recently been examined. Initial observations (Shepherd 1973) on the feeding response as related to water flow indicated some effect, which has since been shown in laboratory studies (Fleming et al. 1997, Higham et al. 1998). Similarly, the preference of these species for areas of high water flow, free from pollution (Shepherd 1973) suggests little tolerance for adverse environmental conditions.

Several aspects of water quality may affect an aquaculture animal's performance, in terms of growth rate, food conversion ratio, disease resistance and flesh quality (Wickins 1981). The most commonly considered aspects are oxygen, ammonia, nitrite, nitrate, pH, carbon dioxide, alkalinity, hardness, suspended solids, and dissolved organic matter (Wickins 1981). Although the most economic water quality levels for overall aquaculture production may be higher than those that are biologically desirable (Wickins 1981), it is still necessary to have quantifiable information on the affect of water quality variables on these animals. The estimation of 'safe' concentrations of water quality variables is most robust and useful for commercial operations.

Testing the toxicity of substances on organisms involves the use of bioassays. Bioassays are usually carried out as a means of determining the no-adverse-biological effects concentration of a chemical (Cairns and Pratt 1989). These can be either lethal, acute bioassays or sublethal, chronic bioassays. The importance of 'safe' concentrations for aquaculture, combined with the relative ease of performing an acute bioassay, meant that in many past situations, acute, lethal bioassays were used to predict safe concentrations for growth (Buikema et al. 1982). The use of arbitrary Application Factors (AF) for this purpose has now fallen from favour. In the absence of chronic toxicity data, acute bioassay data converted via an AF was a useful means for predicting safe concentrations. However, as acute bioassays are a measure of the lethal nature of toxicants and chronic bioassays are a measure of growth limitations, it is difficult to reconcile the AF-derived data with experimental results from chronic bioassays, once the latter are available (Giesy and Graney 1989).

The relationship of water quality components to culture animals can usually be expressed on a continuum. The response to water quality components such as ammonia and nitrite normally involves an initial level of tolerance and a subsequent decline in tolerance with increasing concentration. Another response to water quality components such as temperature, pH and salinity involves a region of tolerance, with declining tolerance beyond each limit. Oxygen content of water also generates a typical response pattern, of a region of tolerance, which declines slowly with decreasing concentration and more rapidly with increasing concentration. (Tomasso 1996). In the following chapters, where possible, experimental data modelling will follow these patterns.

The modelling of chronic toxicity data to determine estimated concentrations where growth reductions will occur was reported by Wickins (1976) as EC values. This approach is directly applicable to commercial situations where the balance of growth reductions against pumping costs may determine the overall conditions at which culture is conducted. However, these commercial considerations require the biological data so that informed management decisions can be made.

Many changes can occur to the physiology and biochemistry of aquaculture animals in adverse conditions (Meyers and Hendricks 1985) and also to their morphology (Mallat 1985). The development of sub-lethal toxicity tests has shown that organisms exhibit stress responses to low, chronic levels of environmental contaminants (Willows 1994). Recent attempts to define stress tests for Australian abalone have focussed on more immediate observations, including salinity tolerance of nutritionally compromised animals (Boarder 1997). The maintenance of constant internal ion concentration is essential and requires active regulation of water influx and ion efflux in aquatic organisms (Mayer et al. 1989). Thus, ion levels in blood or haemolymph have potential as sensitive indicators of chemical exposure. In addition, the use of histopathology to study exposed animals may serve to provide a more complete and accurate description of the effects of a chemical agent (Meyers and Hendricks 1985). Ideally, the development of a suite of indicators for abalone health would benefit the abalone culture industry.

As an aquaculture species, abalone possess qualities such as good texture and taste (Oakes and Ponte 1996), however their biological differences to many other aquaculture species give them a unique place in the market (Oakes and Ponte 1996). In addition, the biology of abalone has similarities with many crustaceans (penaeids, freshwater crayfish) in that all possess haemocyanin as the respiratory pigment (Bonaventura and Bonaventura 1983). Research on the effects of toxicants on the haemocyanin of some of these species has shown altered oxygen-carrying capacity, and hence respiration rate (Chen and Lin 1992, Jensen 1995).

Measurement of oxygen uptake is a critical factor in assessments of stress in aquatic organisms (Beitinger and McAuley 1990, Willows 1994). Generally, oxygen uptake is directly related to metabolic rate and has been widely used to help indicate the health of animals and their overall energy expenditure or activity levels (Innes and Houlihan, 1985). Oxygen uptake may indicate an animal's capacity for growth (which may be expressed as the balance between energy uptake, as food, and energy expenditure) or metabolic adaptations to the variety of environments encountered by both terrestrial

and aquatic organisms (Jobling 1981, Costa 1988, Storey and Storey 1990) including gastropods (Houlihan and Allan 1982). Determining the oxygen uptake rate of abalone from compromised conditions may allow some insights into the metabolism of these animals, and the effects of different toxicants.

In order to determine the environmental requirements of greenlip abalone, the following aims were developed:

- to determine the safe levels of NH_3, NO_2, Dissolved Oxygen (DO) and pH for abalone;
- to examine the respiration rate of abalone at the end of each trial;
- to determine the lethal concentration of NH_3 and if the preceding nutritional history affects tolerance to NH_3;
- to investigate the histopathology of these toxicants on abalone, including tissue histology and haemolymph ionic levels.

2 GENERAL MATERIALS AND METHODS

A description of the experimental systems used in these experiments was presented in poster format:

Maguire, G.B., Edwards, S.J., Hindrum, S.J., Harris, J.O. and Bellchambers, L.M. Bioassay, digestibility and respirometry systems for use with juvenile greenlip abalone, Haliotis laevigata. Presented at the Third International Abalone Symposium; Biology, Fisheries and Culture. Monterey, 26-31 October 1997.

The juvenile greenlip abalone used in these experiments were obtained from a commercial hatchery at Bicheno, Tasmania, Australia, where the research was conducted (E148'18", S41' 53"). The juvenile blacklip abalone used in the pH bioassay were approximately 12 months old and were obtained from a commercial farm at Swansea, Tasmania, Australia. For 2-3 months before experimentation, all abalone were maintained on a mixture of formulated abalone feed and benthic diatoms. Abalone were relaxed using aerated warm water (23-25°C) until they could be easily removed from tank surfaces, or anaesthetised using Benzocaine according to Hahn (1989). Research is currently being conducted into the effects of anaesthetics on greenlip abalone (S. Edwards, pers. comm.). Subsequently, they were weighed to the nearest 0.01 g, measured with callipers to 0.1 mm, tagged (Hallprint, Adelaide, Australia) and randomly distributed to the bioassay units. Initial weight and length data for all experiments are given in Table 2.1 (see Appendix 1).

2.1 Bioassay system

Seawater from an exposed coastline, free from freshwater run-off, was filtered through a commercial sand filter and delivered to six 1100 l reservoirs. Each reservoir was connected to a constant head chamber (150 mm diameter, vertical PVC pipe, operating volume 30 l) which supplied constant flow to three bioassay chambers via standard lengths of black 4 mm polypropylene tubing that entered individual cages within the bioassay tanks (Figure 2.1). These lengths of tubing were replaced, on average, fortnightly. These tanks were cylindrical with a conical base to concentrate solid wastes and a conical top to reduce the air-water interface area (Boyd and Watten 1989). In each 70 l bioassay tank, there were one or two cages (Figure 2.2) (100 mm x 35 cm PVC tube with 6 mm mesh floor and 8 mm mesh wall sections) suspended vertically, containing abalone (Figure 2.3). The initial experiment on ammonia toxicity was conducted at ambient temperatures, while latter experiments used 200 and 300 W aquarium heaters in the bioassay tanks and constant head chambers, respectively, to maintain relatively uniform daily temperatures. In the final, pH bioassay, identical 5 W submersible pumps were placed in each tank to stimulate similar current flow (8.7 $l.min^{-1}$ output at zero head) (Figure 2.4).

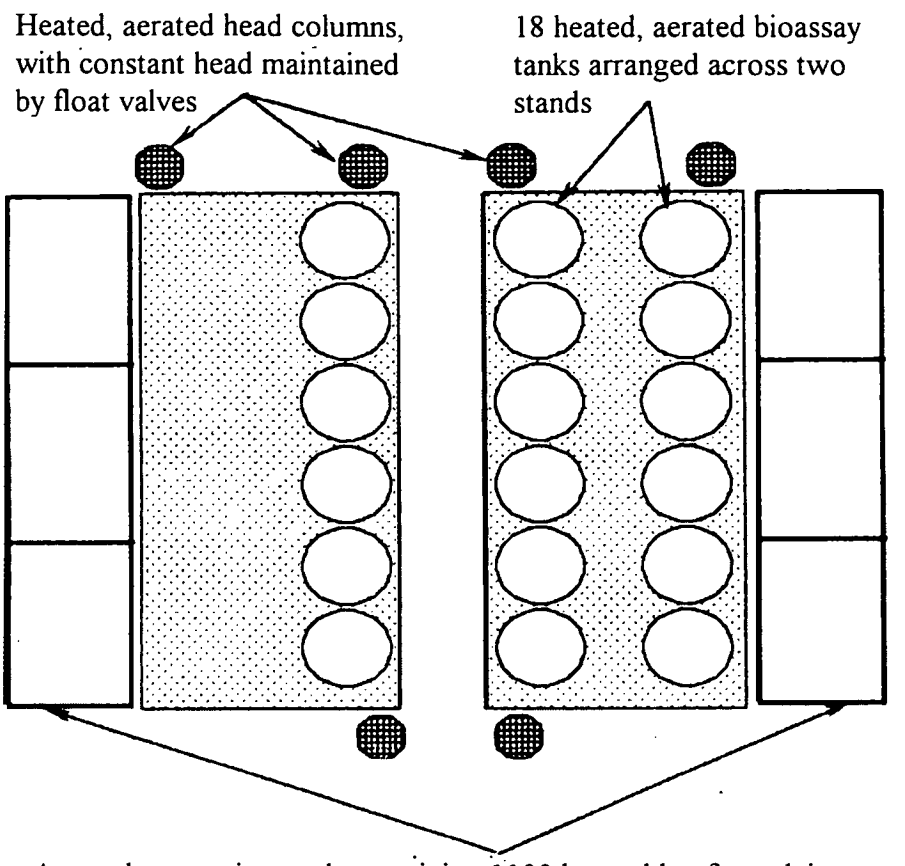

Figure 2.1a Bioassay system. Plan view of bioassay system.

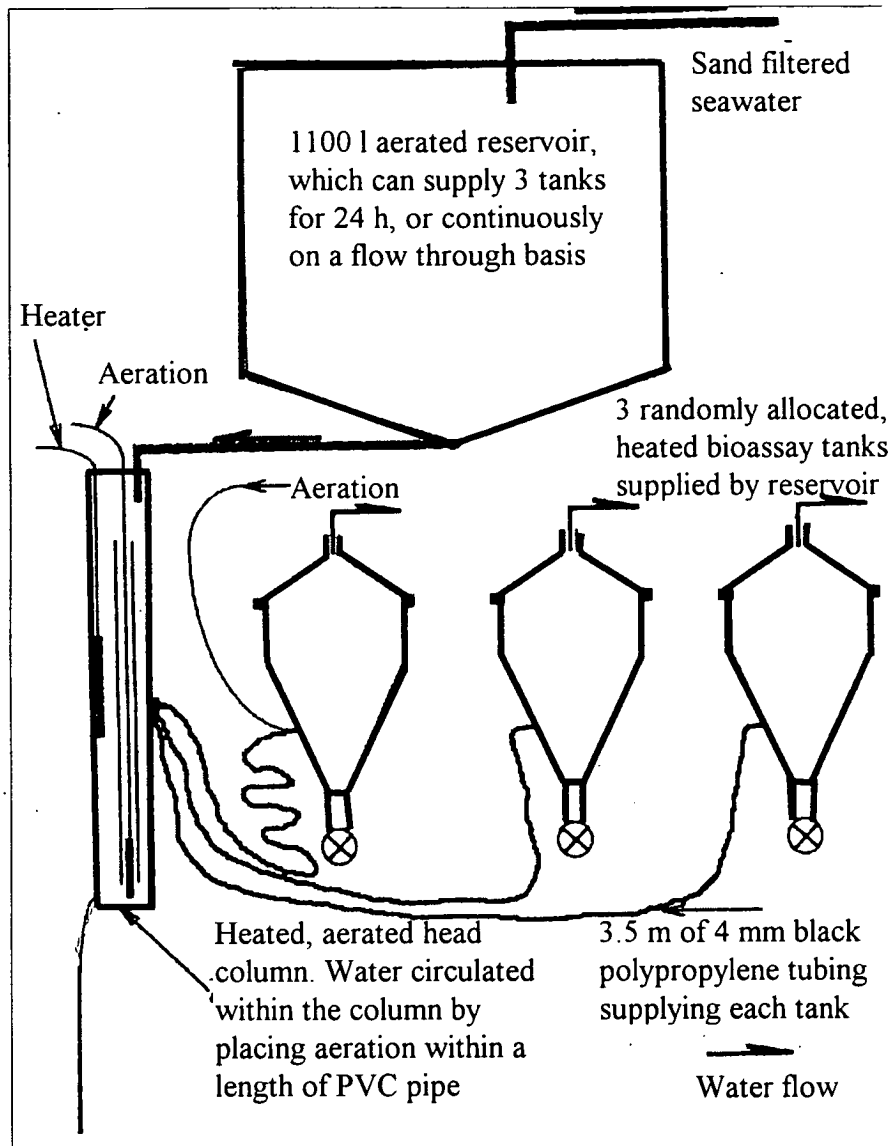

Figure 2.1b Bioassay system. Schematic diagram of bioassay system flow distribution

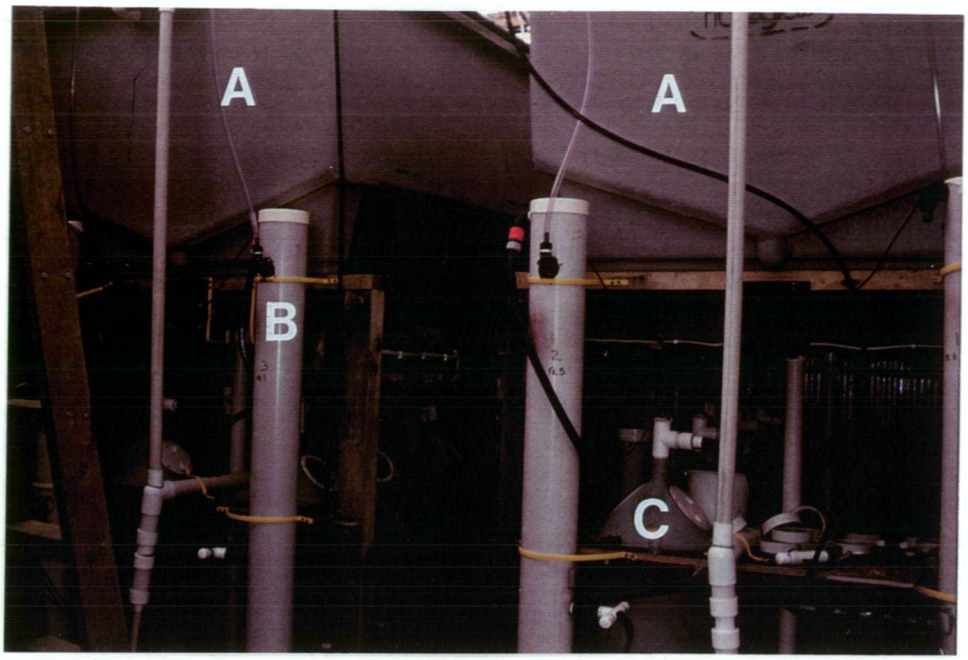

Figure 2.1c Bioassay system. A- Reservoirs (6) on top; B- constant head adjustment columns; C- bioassay tanks supported underneath the reservoirs.

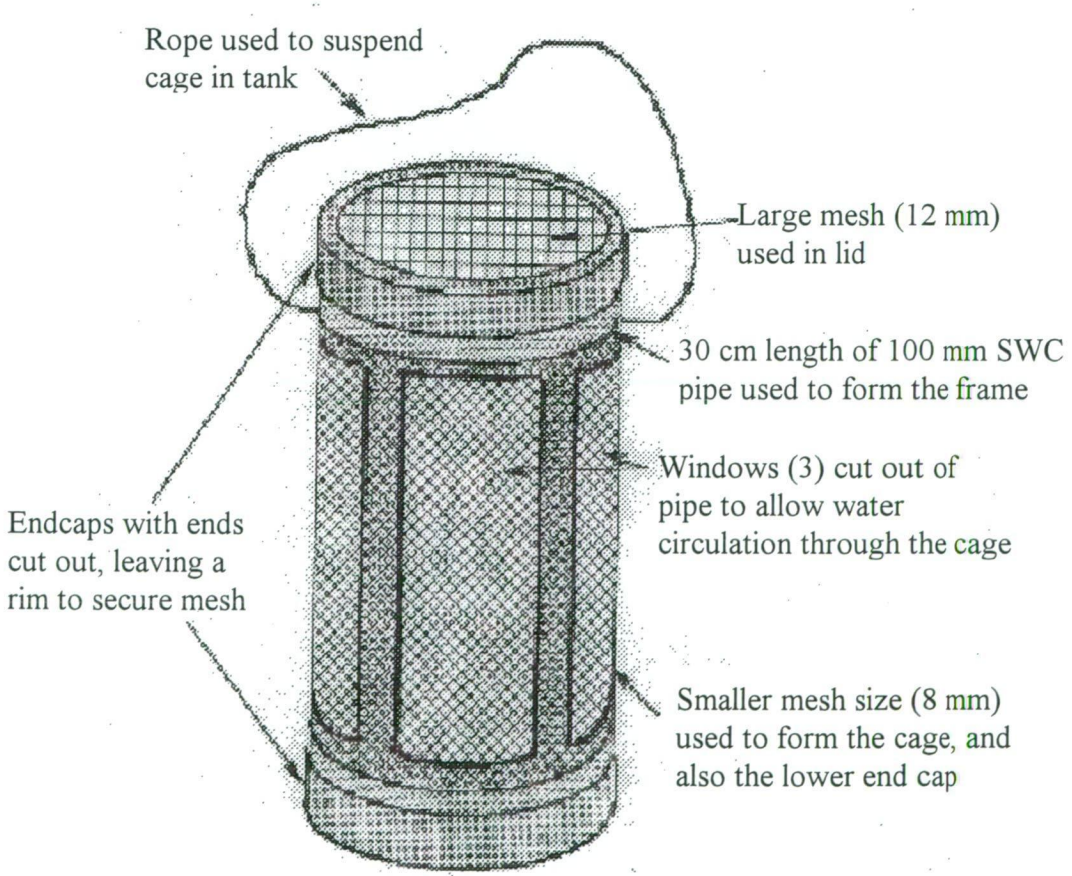

Figure 2.2 Diagram of cage used for housing abalone within bioassay tanks.

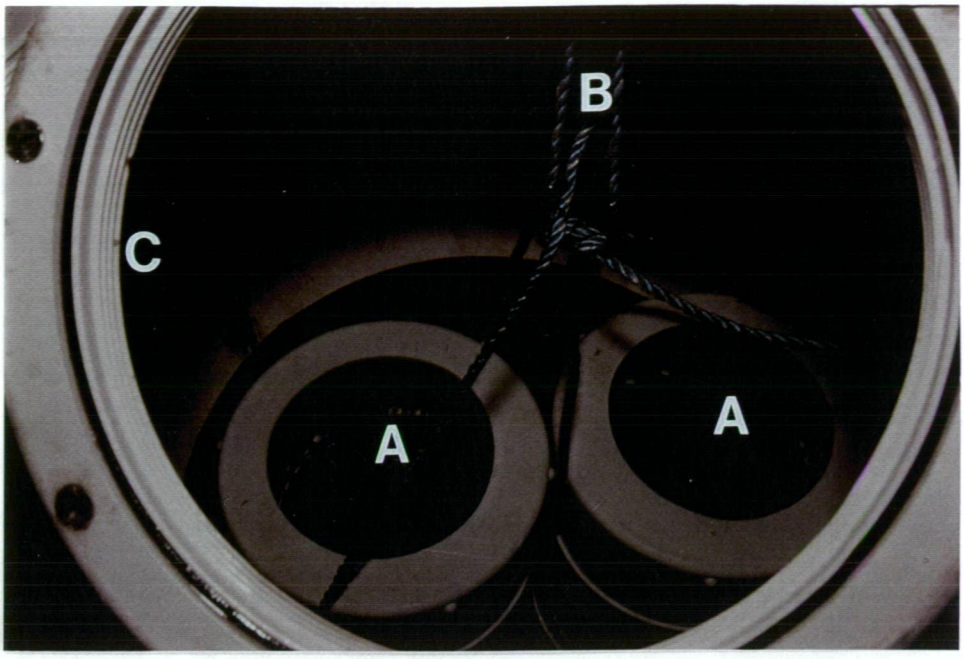

Figure 2.3 Cages arranged within bioassay tank. A- Cages; B- rope for adjusting cage height in tank; C- access hatch.

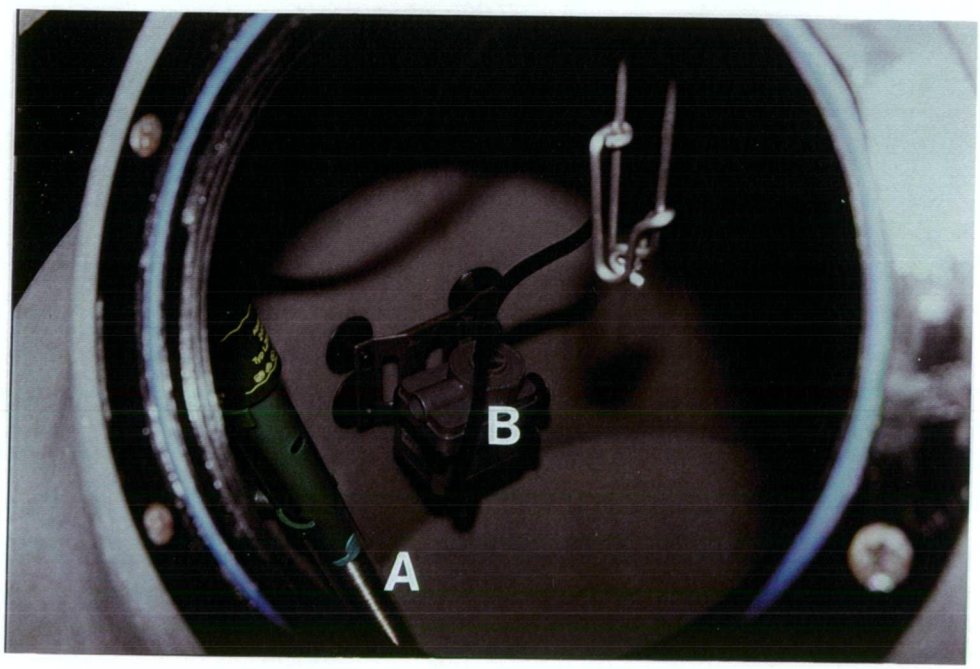

Figure 2.4 Arrangement of immersion heater and submersible pump in a bioassay tank. NB : Heaters were used in all bioassays apart from chronic ammonia toxicity, and the pumps were only used in the chronic pH bioassay. A- 200 W aquarium heater; B- submersible pump (8.7 $l.min^{-1}$ output at zero head).

2.2 Water quality analyses

A WTW Microprocessor Oximeter 0XI 96 oxygen electrode, used for daily measurements, was calibrated before use in 'air-saturated' seawater. The efficiency of this calibration was validated daily by Winkler titration during the oxygen bioassay, and occasionally during all other experiments. Water samples were collected in acid-washed glassware, and ammonia was measured using the indophenol blue spectrophotometric method (Solórzano 1969, as modified by Dal Pont et al. 1974). The concentration of ammonia was measured as total ammonia-nitrogen (TAN), while free ammonia-nitrogen (FAN) was calculated from appropriate temperature, pH and salinity tables (Bower and Bidwell 1978). A pH meter and combination glass electrode (Hanna Instruments, model HI 9025; accuracy @ 20°C ±0.01 pH units, ±0.5°C) were calibrated with phosphate (pH=7.00) and borate (pH=9.28) buffers daily before use (Bruno and Svoronos 1989). Nitrite was measured using the diazotisation method (Grasshoff 1989). Salinity was measured occasionally using a hydrometer, and using the TPS conductivity meter for the pH bioassay.

2.3 Feeding and food consumption

All abalone were fed a proprietary, formulated abalone diet (ABCHOW; 35% protein on a dry matter basis) every two to three days. The feeding ration was adjusted in response to food consumption data as each trial progressed. Food consumption was estimated from uneaten food removed from the base of the cages after two days and dried for 24-48 hours at 55-60°C. Residual food weight was not corrected for soluble and particulate nutrient losses over the two days (Appendix 3). Apparent food consumption (amount of food supplied minus residual food, expressed as g dry weight) was divided by the initial tank biomass, less the weights of any mortalities to that point. All cages were checked daily for abalone mortality.

2.4 Cleaning

A valve in the base of each bioassay tank was opened daily to remove organic wastes. Tanks were cleaned more thoroughly every 6-15 days. Cleaning involved lowering the water level, siphoning enough water from the bioassay tank into a 20 l bucket to cover the cages, removing cages to the bucket, draining the tank, scrubbing the tanks and cages, refilling the tanks directly from the reservoirs and returning the cages to the tanks. This took less than 10 minutes for any tank. The DO level was measured in the buckets to determine if the cleaning process exposed the abalone to altered conditions. DO concentrations within the buckets did not differ by more than 11% (Chapter 5) from the tanks in any of the bioassays.

2.5 Oxygen consumption

At the end of each chronic bioassay, abalone from the bioassay system were transferred to respirometer chambers for three days. These animals had been fed before removal. Abalone that did not attach to transferable plastic strips in the cages within the bioassay units were removed manually, either by sliding them directly from the substrate or by inserting a thin, plastic card underneath each abalone's foot. Temperature and pH levels were measured.

The respirometer system used included five elliptical perspex chambers (2.3 l) normally set up with two replicate chambers for each treatment (15-30 animals per chamber) and one chamber as a control without animals. Water flowing continuously from each 1100 l reservoir was regulated prior to entering each chamber near the base. Flow rates were measured manually twice daily. Flow exiting the top of each individual chamber was diverted by solenoids to either waste (50 min each hour) or to a flow cell containing an Orion oxygen electrode (10 min each hour) for data recording. The system automatically cycled through sampling the flow from each chamber, and after sampling the five chambers, the remaining 10 min in each hour was used to automatically calibrate the electrode using fully aerated seawater. The overall design of the system is similar to that used by McLean and Tobin (1987) for terrestrial organisms. Data from the oxygen electrode was collected by a datalogger every second, averaged every minute and downloaded to a computer at the end of each

experiment. The datalogger also stored water temperature data from thermocouples placed throughout the system. Final mV output was converted to the data presented using a LOTUS spreadsheet where drift between calibrations was assumed to be linear (for both flow and oxygen). The amount of oxygen used in each chamber was calculated as the percentage of the full saturation value from mV output. Oxygen consumption data for chambers containing animals were corrected for the oxygen uptake of the control chamber and the final oxygen concentration divided by the wet weight of animals to provide mg $DO.kg^{-1}.h^{-1}$. The data presented are average values over the 24 hours during the third (and last) day of each respiration trial.

2.6 Statistical analysis

For chronic bioassays, data were subjected to one factor ANOVA after meeting assumptions of normality using the Shapiro-Wilk test (Zar 1996) and homogeneity of variance using Cochran's test (Underwood 1981). All analyses were performed on data from the entire duration of each chronic exposure trial. Replicates were considered to be independent and ammonia, nitrite, dissolved oxygen concentration and pH levels were analysed as fixed factors for each analysis. Survival data and whole wet body weight: shell length ratios were transformed ($\sqrt{\text{arcsin}}$ and log, respectively) prior to analysis. Results for each treatment concentration were compared against data for the control using Dunnet's test (Sokal and Rohlf 1995). Preliminary analysis in each experiment indicated that initial size did not affect growth rate. The size ranges of abalone used for each experiment are reported in each chapter and Table 2.1, with all analyses, including assessment of covariates (Sokal and Rohlf 1995), conducted using JMP 3.0 software (SAS Institute). Regressions are based on data for each replicate rather than treatment means. Curve fitting was conducted on Sigmaplot using the least squares method.

Table 2.1 Initial lengths and weights of all abalone used in bioassays for this study.

Variable	N	Length					Weight				
		Mean	SD	SE	Min	Max	Mean	SD	SE	Min	Max
Ammonia	953	31.81	4.22	0.14	22.6	42.6	4.48	1.89	0.06	1.39	11.49
Nitrite	719	34.97	3.37	0.13	28.3	44.2	5.61	1.70	0.06	2.61	11.09
DO	603	44.10	4.31	0.18	30.7	52.9	10.75	3.03	0.12	3.78	18.64
Ammonia/ diet	96	56.24	6.22	0.63	38.8	71.2	22.35	6.48	0.66	7.80	39.65
pH -greenlips	561	26.49	2.83	0.12	19.9	34.5	2.30	0.73	0.03	1.01	5.13
pH - blacklips	559	22.92	2.92	0.12	17.0	34.5	1.56	0.64	0.03	0.54	5.00

3 EFFECT OF AMMONIA ON THE GROWTH RATE AND OXYGEN CONSUMPTION RATE OF JUVENILE GREENLIP ABALONE.

This chapter is published as :

Harris, J.O., G.B Maguire, S.J. Edwards, S.M. Hindrum 1998. Effect of ammonia on growth rate and oxygen consumption rate for juvenile greenlip abalone, *Haliotis laevigata* Donovan. Aquaculture, 160(3/4):259-272.

3.1 Introduction

Ammonia is the principal nitrogenous compound excreted by aquatic animals (Colt and Armstrong 1981) and being toxic to fish, crustaceans and molluscs, can limit production in aquaculture (Epifanio and Srna 1975, Wickins 1976, Russo 1985, Allan et al. 1990, Russo and Thurston 1991). In solution, ammonia exists in a pH and temperature-mediated equilibrium between the unionised and ionised forms, with unionised ammonia considered more toxic (Russo and Thurston 1991). Ammonia induces detrimental changes in tissue structure, cell function, blood chemistry, osmoregulation, disease resistance, growth and reproductive capacity (Colt and Armstrong 1981, Russo 1985, Jeney et al. 1992). Chronic exposure can result in the deterioration of several physiological functions, any one of which may be the ultimate cause of death (Russo 1985).

Measurement of oxygen uptake is a critical factor in assessments of stress in fishes (Beitinger and McAuley 1990). Generally, oxygen uptake is directly related to metabolic rate and has been widely used to help indicate the health of animals and their overall energy expenditure or activity levels (Innes and Houlihan 1985). Oxygen uptake may indicate an animal's capacity for growth (which may be expressed as the balance between energy uptake, as food, and energy expenditure) or metabolic adaptations to the variety of environments encountered by both terrestrial and aquatic organisms (Jobling 1981, Costa 1988, Storey and Storey 1990) including gastropods (Houlihan and Allan 1982). Ammonia may affect gill structure (Smart 1976), respiratory function (Chen and Lai 1992, Chen and Lin 1992, Chen and Lin 1995, Knoph 1996), and oxygen consumption (Smart 1978) in aquatic animals.

Abalone culture is expanding in several countries in response to declining fisheries production (Hone and Maguire 1996). As production increases, the reliance on protein-rich, formulated feeds and the introduction of recirculating culture systems increase the likelihood of exposure to elevated ammonia concentrations. Little information is available to farmers on the toxicity of ammonia to abalone and marine gastropods in general, and in particular, the effect on growth. In this study we

assessed the chronic toxicity of unionised ammonia in terms of growth rate, food consumption rate and oxygen consumption rate for juvenile greenlip abalone, *H. laevigata*, the most widely farmed abalone in Australia.

3.2 Materials and methods

The juvenile greenlip abalone were approximately three years old. The mean length and weight of the abalone were 31.8±4.2 mm and 4.48±1.89 g (n=953) (mean±SD) Abalone were anaesthetised (0.1% benzocaine) until they could be easily removed from the tank surfaces, weighed, measured and randomly distributed to 18 bioassay units (50 per unit). Abalone were exposed to specific ammonia concentrations for 95 days, then weighed and their length measured in order to calculate specific growth rate (SGR). Abalone that died during the experiment were replaced (minimum exposure period to ammonia in the bioassay system was 58 days).

3.2.1 Bioassay system

The six 1100 l reservoirs were drained, cleaned and refilled each day with seawater dosed with the appropriate amounts of NH_4Cl. In each 70 l bioassay tank were two cages suspended vertically, each containing 25 abalone. Daily flow rates averaged 165±20 ml.min^{-1} (n=90; 18 tanks on 5 occasions), giving an effective replacement rate of 90 % of bioassay tank volume in 12-15 hours. This was slightly less than that recommended for aquatic toxicological studies by Sprague (1969) of 90% replacement in 8-12 hours. Clearly, replacement rate, as a percentage of cage volume (1 l per cage) was much higher. The experiment was conducted at ambient temperature of 16.9 ±1.1°C (n=82) (range 13.3-19.6°C). Some ammonia was expected to be removed within the system, through bacterial and chemical means. The average daily recovery of TAN between reservoirs and bioassay tanks varied from 82.0-98.5% (n=5).

3.2.2 Water quality analysis

The pH, temperature and dissolved oxygen in all tanks, along with the ammonia concentration of one replicate tank from each treatment (and occasionally all 18 tanks), were measured on most days (Table 3.1). Most ammonia samples were measured immediately after collection, otherwise they were frozen for up to seven days and subsequently thawed (Degobbis 1973). Nitrite was measured occasionally, using the diazotization method (Grasshoff 1989).

3.2.3 Experiment 3.1 : Chronic ammonia exposure

One control and five experimental treatments were established (Table 3.1); average ammonia concentrations ranged from 0.006 - 0.188 mg FAN.l^{-1} (0.24 - 9.04 mg TAN.l^{-1}). The abalone were acclimatised to the bioassay system for 5-6 days before the addition of NH_4Cl. The concentrations were established abruptly, increasing from ambient seawater to the experimental concentrations. All cages were checked daily for mortality. From the beginning of the experiment to day 23, the ammonia concentration in treatment 6 was 0.363±0.024 mg FAN.l^{-1}. This level was reduced to an average of 0.159±0.016 mg FAN.l^{-1} until the end of the experiment, in order to reduce mortality. On day 20, 43 abalone were added to replace losses in treatments 3, 5 and 6 (see Appendix 2). These abalone were anaesthetised, tagged, weighed and measured as above.

The feeding ration was adjusted in response to food consumption data, estimated on five occasions (days 22, 32, 68, 70, 73).

Cages were without aeration until day 5, when aeration lines were installed. Tanks were cleaned, on average, every six days.

3.2.4 Experiment 3.2 : Oxygen consumption rates (post bioassay)

On day 81 of the growth trial, 35 and 48 abalone from the control and treatment 6 respectively were transferred to respirometer chambers for three days. These animals had been fed prior to removal. Ammonia concentration, pH and temperature levels of

effluent water from the reservoirs were measured daily to determine FAN concentrations (Table 3.2). On day 84, 41 and 40 abalone from two replicates of treatments 2 and 3 respectively, were transferred to the respirometer for three days, and on day 87, 48 and 37 abalone from two replicates of treatments 4 and 5 respectively, were transferred to the respirometer for three days.

3.2.5 Statistical analysis

The EC_5 and EC_{50} values, those ammonia concentrations where growth rate was reduced by 5 and 50%, respectively, were estimated from two intersecting linear regressions (Sedgwick 1979, Maguire and Hume 1982).

3.3 Results

3.3.1 Experiment 3.1 : Chronic ammonia exposure

In terms of shell length, growth rate declined with increasing ammonia concentration over the entire experimental range (Figure 3.1). Growth rate relative to the control (0.006 mg FAN.l^{-1}) was not significantly reduced ($p>0.05$) from 0.025-0.031 mg FAN.l^{-1}, however, at 0.054-0.188 mg FAN.l^{-1} significant growth rate reductions occurred ($p<0.05$). In terms of whole wet body weight gain, no significant ($p>0.05$) reduction in growth rate occurred from 0.025 to 0.054 mg FAN.l^{-1}, however, significant ($p<0.05$) reductions occurred from 0.110-0.188 mg FAN.l^{-1} (Figure 3.2). The EC_5 and EC_{50} values from the weight data were 0.041 and 0.158 mg FAN.l^{-1} (1.30 and 3.92 mg TAN.l^{-1} at 18°C and pH = 8.2), respectively (Figure 3.2). Survival was high in all but the highest treatment (0.188 mg FAN.l^{-1}), where a significant ($p<0.001$) reduction occurred (Table 3.1). The initial mortalities observed within the highest treatment (0.363±0.024 mg FAN.l^{-1} up to day 23 (range 0.291-0.454 mg FAN.l^{-1})) resulted in this treatment requiring 77% of the replacement abalone.

Food consumption declined with increasing ammonia concentration (Figure 3.3), however, significant declines in food consumption ($p<0.001$) relative to the control occurred only in treatments 5-6 (0.110-0.188 mg FAN.l^{-1}).

Nitrite concentrations in the bioassay tanks were significantly higher ($p<0.001$) for treatments 2-6 than for the control (0.006 mg FAN.l^{-1}) (Figure 3.4). Covariance analysis indicated that background nitrite concentration did not significantly influence differences in growth rates at least within treatments ($p>0.05$). This analysis relies on there being considerable variation among replicates in terms of growth rate and nitrite concentration, which is evident in Figures 3.1, 3.2 and 3.4. pH levels for treatments 3-6 were significantly different to the controls ($p<0.05$)

3.3.2 Experiment 3.2 : Oxygen consumption rates (post bioassay)

The oxygen consumption data indicated a rapid increase in oxygen consumption with increasing ammonia concentration up to 0.235 mg FAN.l^{-1} (Figure 3.5). The quadratic model suggests a decline in oxygen consumption past this level, but without more data points, this cannot be presumed. No significant increase ($p>0.05$) in oxygen consumption rate occurred from 0.0007-0.022 mg FAN.l^{-1}, however from 0.073-0.418 mg FAN.l^{-1} significant increases ($p<0.05$) in oxygen consumption rate occurred.

Table 3.1 Water quality parameters within the chronic ammonia exposure trial (Experiment 3.1)[1].

Treatment	FAN mg.l^{-1}			TAN mg.l^{-1}			pH	% survival[2,3,4]
	Mean±SE[2]	Min	Max	Mean±SE[2]	Min	Max	Mean±SE[2]	
1	0.006±0.001	<0.005	0.027	0.237±0.02	0.220	1.01	7.99±0.02	98.67±1.33[a]
2	0.025±0.005	<0.005	0.062	1.01±0.16	0.155	10.73	7.93±0.01	99.33±0.67[a]
3	0.031±0.002	0.007	0.073	1.46±0.09	0.405	4.04	7.88±0.02	98.18±1.82[a]
4	0.054±0.005	0.007	0.129	2.65±0.18	0.405	7.00	7.84±0.03	100±0[a]
5	0.110±0.009	0.021	0.316	6.16±0.32	1.625	15.17	7.78±0.01	95.11±2.91[a]
6	0.188±0.016	0.013	0.455	9.04±0.47	0.895	19.4	7.84±0.02	49.58±5.43[b]

[1] Average water temperature, salinity and dissolved oxygen were 16.9 ±0.1°C (n=82) (range 13.3-19.6°C), 34.0‰ and 7.6±0.8 mg.l^{-1} (range 5.3-9.2; n= 67)

[2] Values are means±SE (n=3) for each treatment.

[3] Data were transformed prior to statistical analyses.

[4] Means sharing a common superscript are not significantly different (p>0.05).

Table 3.2 Water quality parameters in respirometer chambers (Experiment 3.2)[1,6].

Treatment	Ammonia ($mg.l^{-1}$)		pH[2]	Total wet body weight (g)
	FAN	TAN		
1[3]	0.0005±0.0004	0.014±0.013	8.16	163.14
2[4]	0.022±0.001	0.74±.07	8.08	251.32
3[4]	0.073±0.015	2.44±0.34	8.16	209.21
4[5]	0.140±0.010	5.45±0.23	8.16	268.56
5[5]	0.235±0.029	9.52±0.76	8.08	218.74
6[3]	0.418±0.040	16.0±2.31	8.04	221.88

1. Values are means±SE (n=2-3) for each treatment
2. Values are -log of treatment mean $[H^+]$ concentration.
3. Average experimental flow rate = 141.8 $ml.min^{-1}$.
4. Average experimental flow rate = 130.5 $ml.min^{-1}$.
5. Average experimental flow rate = 131.0 $ml.min^{-1}$.
6. Average water temperature was 15.5±0.2°C.

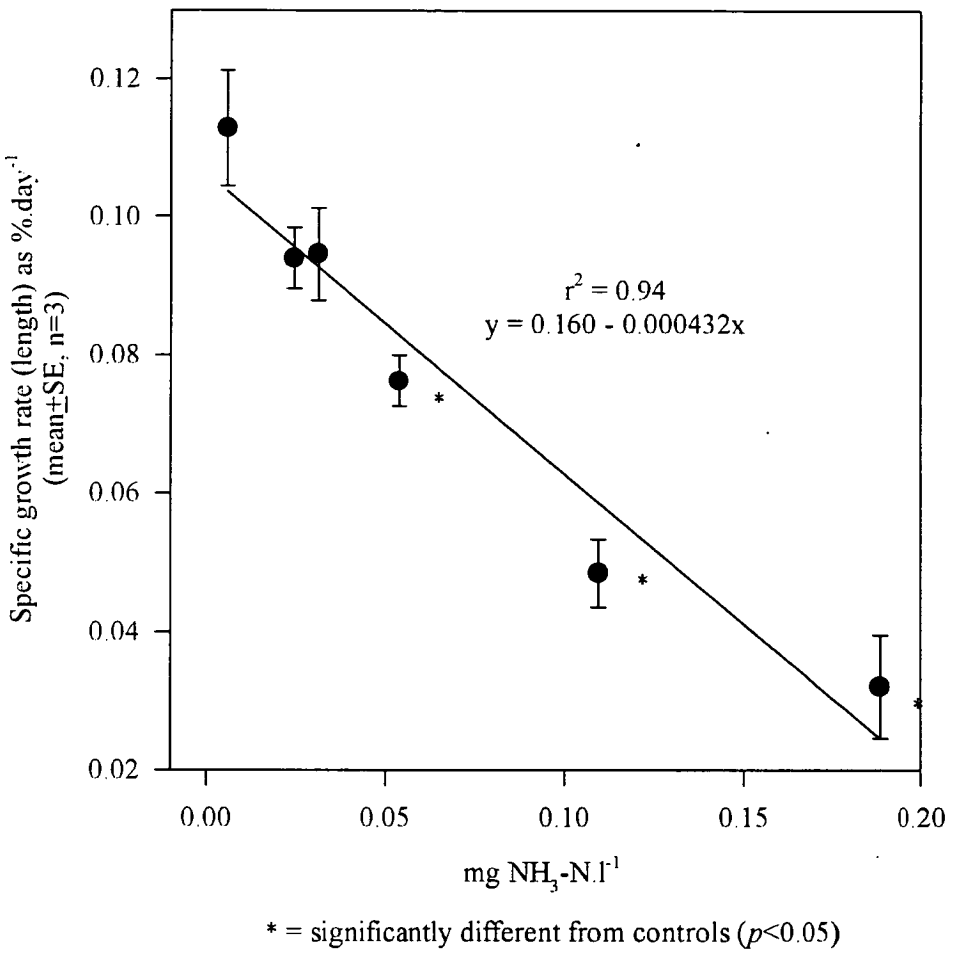

Figure 3.1 Specific growth rate (length) of juvenile greenlip abalone, *Haliotis laevigata*, exposed to ammonia in Experiment 3.1.

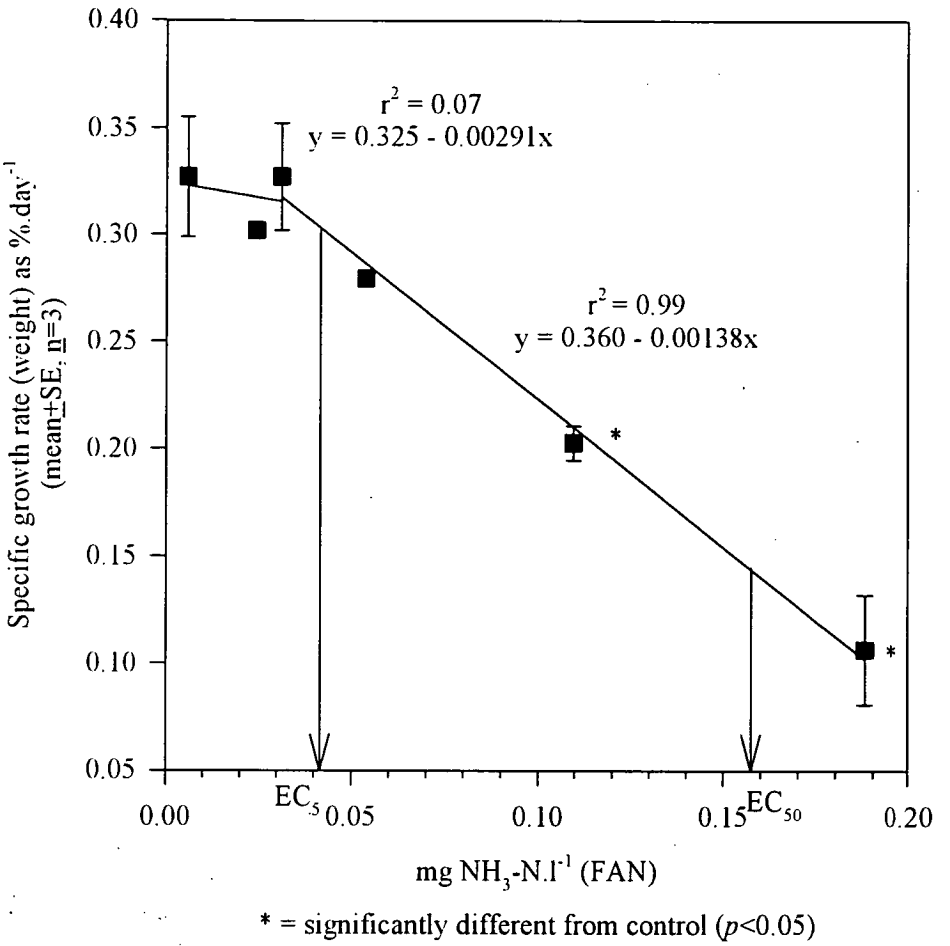

Figure 3.2 Specific growth rate (whole weight) of juvenile greenlip abalone, *Haliotis laevigata*, exposed to ammonia in Experiment 3.1.

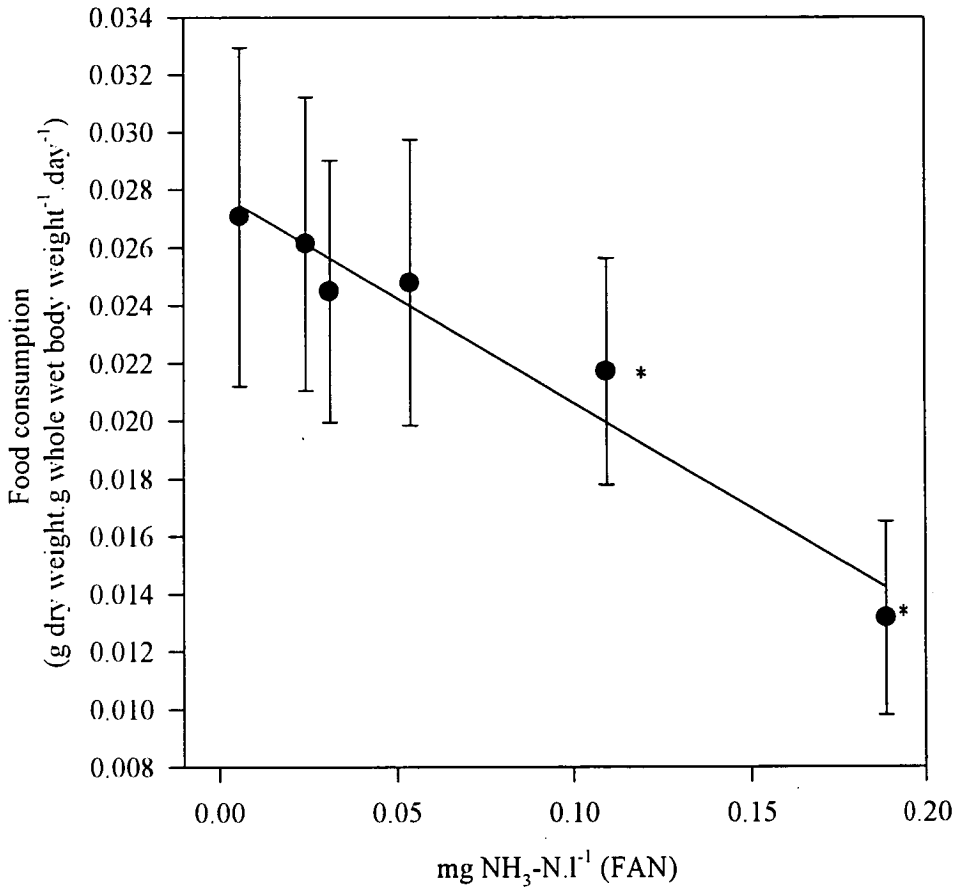

Figure 3.3 Food consumption (g.g^{-1}.day^{-1}) of juvenile greenlip abalone, *Haliotis laevigata*, exposed to ammonia in Experiment 3.1, based on four, two day feeding cycles (mean±SE; n = 3).

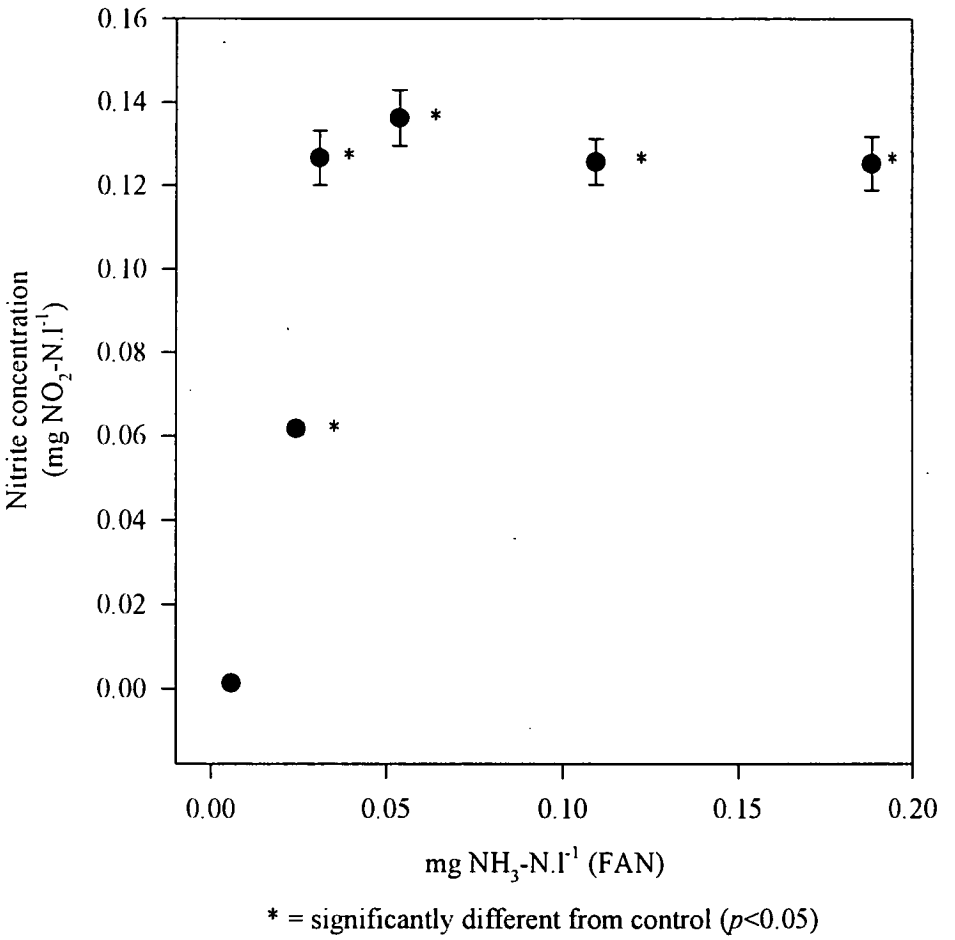

Figure 3.4 Concentration of nitrite experienced by juvenile greenlip abalone, *Haliotis laevigata*, during chronic ammonia toxicity trial, based on measurements on three occasions in Experiment 3.1 (mean±SE; n = 3).

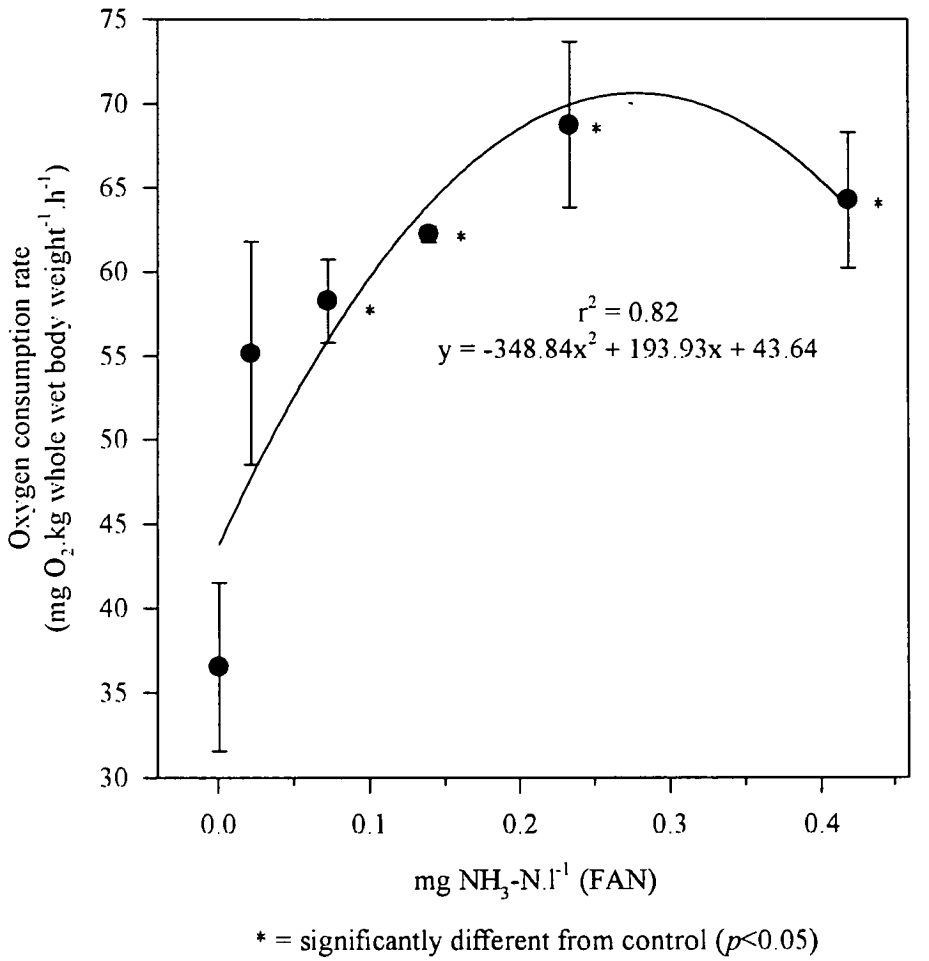

Figure 3.5 Oxygen consumption rate of juvenile greenlip abalone, *Haliotis laevigata*, exposed to ammonia in Experiment 3.2 (mean±SE; n = 2).

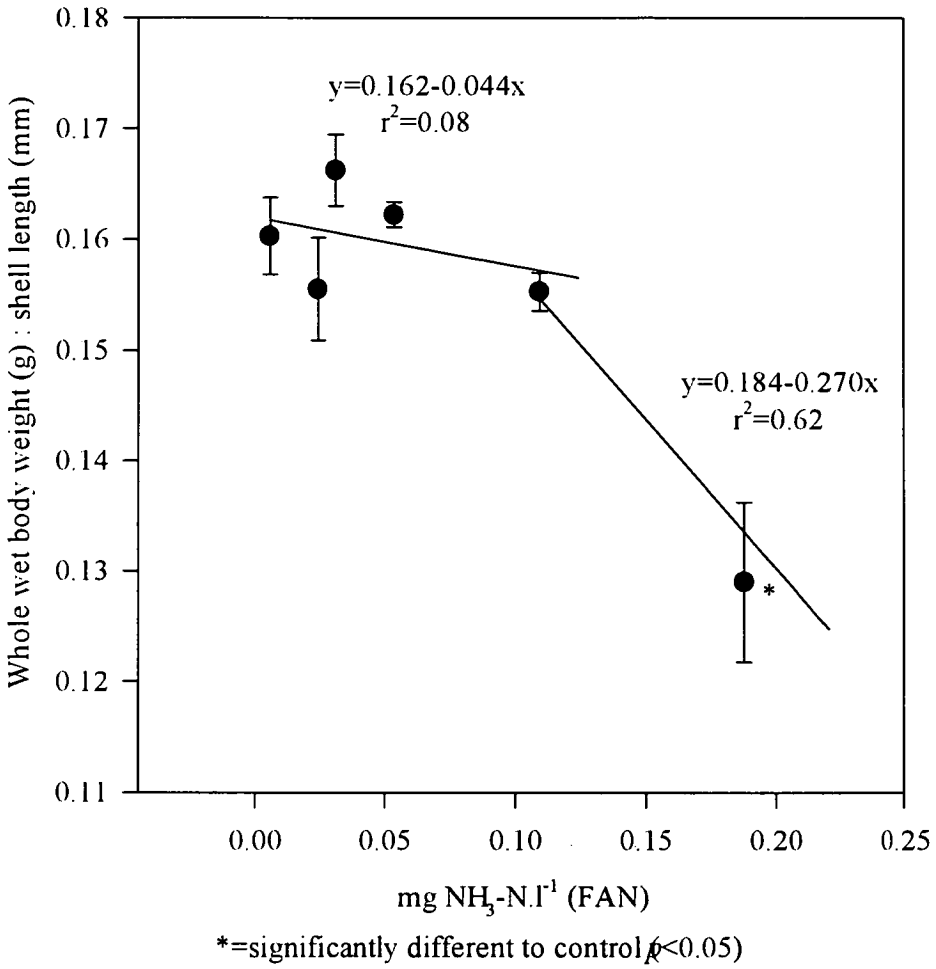

Figure 3.6 Whole wet body weight : shell length ratio of juvenile greenlip abalone, *Haliotis laevigata*, subjected to chronic ammonia exposure in Experiment 3.1 (mean±SE).

3.4 Discussion

Growth rates of control animals (SGR weight = 0.33±0.03 %.day^{-1}; SGR length = 0.11±0.01 %.day^{-1}) were comparable with a concurrent trial with greenlip abalone of a similar size, conducted in outdoor ambient tanks (SGR weight = 0.31±0.03 %.day^{-1}; SGR length = 0.11±0.02 %.day^{-1}) (Maguire et al. 1996a). This suggests that the bioassay environment was not directly stressful for the control animals. However, faster growth rates for this species (SGR weight = 0.8 %.day^{-1}) have been recorded in culture systems at ambient temperatures in Tasmania (Hindrum et al. 1996). The growth rates of the abalone replaced into 0.188 mg FAN.l^{-1} were comparable to the original abalone from this treatment (Appendix 2), and it is considered unlikely that these would unduly bias results for this treatment. Day and Fleming (1992) suggest that for nutritional studies of *Haliotis rubra*, trials longer than 100 days are required. It could reasonably be expected that responses to water quality alterations may manifest sooner than comparable limitations due to nutritional deficiencies.

Colt and Armstrong (1981) predicted significant growth reductions for most aquatic animals between 0.05-0.2 mg FAN.l^{-1}. Significant reduction in length and weight occurred at 0.054 mg FAN.l^{-1} and 0.110 mg FAN.l^{-1}, respectively for *H. laevigata* in our study. Russo and Thurston's (1991) review of ammonia toxicity indicated that sublethal effects in juvenile or adult fish occur in the range 0.04-0.96 mg FAN.l^{-1}. Growth reductions, in terms of weight gain, were observed at 0.89 mg FAN.l^{-1} and 0.78 mg FAN.l^{-1} in school prawns, *Metapenaeus macleayi*, and leader prawns, *Penaeus monodon*, respectively (Allan et al. 1990). Epifanio and Srna (1975) studied the toxicity of ammonia to the bivalve molluscs *Crassostrea virginica* and *Mercenaria mercenaria* in terms of the effect on algal clearance rates, and found a 54% and 9% reduction at a concentration of 0.72 mg FAN.l^{-1}, respectively. However, the ability of these bivalves to close their valves in unfavourable conditions may contribute to their tolerance (Epifanio and Srna 1975). Nevertheless, the levels of unionised ammonia required to depress shell growth in *H. laevigata* are at the lower end of Colt and

Armstrong's (1981) prediction and suggest a greater sensitivity than some other aquatic species.

Growth responses described in Figures 3.1 and 3.2 may indicate that shell growth is more sensitive to ammonia than growth on a whole weight basis. This pattern is also evident in whole wet body weight (WWBW) : shell length (SL) data (Figure 3.6). While shell growth is more sensitive to ammonia at low concentrations, the slopes in the linear models in Figures 3.1 and 3.2 and the decline in the ratio (Figure 3.6) indicate that at high concentrations, whole weight is affected more than shell growth. While product quality is more strongly influenced by body growth than shell growth, the influence of ammonia on shell growth at low concentrations may still be important through a limiting effect on body growth (Palmer 1981).

Unfortunately, elevated nitrite concentrations and reduced pH also occurred during the bioassay (Figure 3.4 and Table 3.1). This problem was thought to arise from the 3.5 m lengths of 4 mm tubing supplying seawater plus ammonia from the reservoirs to the bioassay tanks. Presumably, over extended periods these develop a film of nitrifying bacteria and convert ammonia to nitrite (Wickins 1983). In the latter half of this trial these supply lines were replaced fortnightly to overcome the problem. However, covariance results indicate that nitrite and pH were not significant confounding factors. The decrease in pH observed with increasing ammonia is small and unlikely to influence the experiment, as work with bivalves suggests that pH affects growth when below 7 (Bamber 1990). Subsequent research arising from these results revealed that the pH levels encountered in this experiment were within the EC_5 estimations for greenlip abalone, and are unlikely to have influenced growth rates (Chapter 6). Nitrite is considered to be an unlikely influence on the results, as modelling of growth responses indicates that growth depression increased with ammonia concentration in the range 0.031-0.188 mg FAN.l^{-1} (Figures 3.1 and 3.2), despite relatively constant nitrite levels. The overall levels encountered (maximum = 0.143 mg NO_2-N.L^{-1}) were much lower than the concentrations which produced a 50% growth reduction in juvenile *Penaeus indicus* (6.4 mg NO_2-N.L^{-1}) (Wickins 1976), or which depressed algal clearance rates for the bivalves *M. mercenaria* and *C.*

virginica (280 mg NO_2-N.L^{-1}) (Epifanio and Srna 1975). However, the influence of low levels of nitrite on growth of marine gastropods warranted further attention and led to the research described in Chapter 4.

The decrease in food consumption with increasing ammonia concentration observed for *H. laevigata*, has also been reported for *Haliotis discus hannai* (Sano and Maniwa 1962), although it was only towards the upper limit of our experimental range that significant depression of food consumption occurred. Similarly, a decrease in growth of juvenile turbot, *Scophthalmus maximus*, exposed to ammonia was explained partly by a decrease in food consumption (Rasmussen and Korsgaard 1996).

The respirometry data for *H. laevigata* show an increase at low TAN over the range tested in the growth trial and a decline in oxygen consumption rate above 9.5 mg TAN.l^{-1}. However, we note that the highest concentration tested in the respirometry exceeded the average concentration in treatment 6 of the growth trial. Based on mortality patterns over the first 20 days for treatment 6, the highest concentration in the respirometry would be expected to cause mortality over an extended period. In contrast to our respirometry results, Sano and Maniwa (1962) found that elevated ammonia levels, induced by higher feed inputs, depressed oxygen consumption. However, their experimental design does not allow for this result to be attributed specifically to an effect of ammonia on the oxygen consumption rate of abalone, as food decomposition would have a marked influence on dissolved oxygen levels and other water quality factors. Other studies of penaeid prawns (Chen and Lai 1992, Chen and Lin 1992, Chen and Lin 1995) demonstrated a similar increase in oxygen consumption with increasing ammonia-nitrogen. These studies indicate that for *Penaeus chinensis*, an increase in oxygen consumption rate occurs with increasing ammonia-nitrogen concentration up to 10 mg TAN.l^{-1}, whereas *Penaeus japonicus* exhibited a subsequent decline in oxygen consumption rate above 5.01 mg TAN.l^{-1}. Increased oxygen consumption at high and acute ammonia exposures has also been seen for salmonids (Smart 1978).

In conclusion, greenlip abalone appear highly sensitive to ammonia. The abalone exhibited reduced energy intake as indicated by decreased food consumption but increased energy expenditure, as indicated by oxygen consumption rate, and hence growth was depressed. This influence of ammonia has implications for system design and management, in the use of low protein diets (Jirsa et al. 1997), high water exchange rates, efficient biofiltration and regular removal of organic wastes.

4 EFFECT OF NITRITE ON GROWTH AND OXYGEN CONSUMPTION FOR JUVENILE GREENLIP ABALONE.

This chapter is published as :

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5 EFFECT OF LOW DISSOLVED OXYGEN ON GROWTH RATE AND OXYGEN CONSUMPTION RATE FOR JUVENILE GREENLIP ABALONE.

This chapter is published as :

Harris, J.O., G.B Maguire, S.J. Edwards, D.R. Johns 1999. Low dissolved oxygen reduces growth rate and oxygen consumption rate for juvenile greenlip abalone, *Haliotis laevigata* Donovan. Aquaculture, 174:265-278.

5.1 Introduction

Low DO concentration limits production of many aquaculture species (Boyd and Watten 1989). DO is classified as a limiting factor for growth (Fry 1971, Brett 1979), although it does not act directly on growth as many toxins do, but limits the scope for aerobic metabolism. Aerobic organisms are either oxygen regulators or conformers, with the latter's oxygen consumption being entirely dependent on external concentrations. Oxygen regulators, however, consume oxygen independent of external concentration down to a specific level (*Pc*; critical pressure), below which they behave as oxygen conformers. The region above *Pc* is known as the zone of respiratory independence, and that below *Pc* is termed the zone of respiratory dependence (Fry 1971). The energy cost to oxygen conformers in dealing with declining oxygen consumption can severely limit growth (Ingerson and Geddes 1995) and impact on survival if unchecked. Abalone appear to be oxygen regulators, although starvation is known to limit regulatory ability (Jan and Chang 1983, Gaty and Wilson 1986). The total energy expenditure of oxygen regulators declines in accordance with DO concentration in the zone of respiratory dependence, with anaerobic metabolism providing partial compensation for reduced energy (Herreid 1980).

In certain aquaculture situations, oxygen concentrations can vary markedly. This is particularly evident during phytoplankton blooms (Elston 1983). However, it can also be variable in systems subject to high biological oxygen demand (BOD) in which uneaten food and decaying wastes are only removed intermittently, as in some abalone production systems where phytoplankton blooms are unlikely (Hindrum et al. 1996). Under conditions of high BOD, DO levels may decline below *Pc* into the zone of respiratory dependence for the cultured animal and limit the scope for aerobic metabolism (Willows 1994).

Little information is available on the effect of hypoxia on growth of molluscs, especially abalone, although several studies are available on the effect of hypoxia on oxygen consumption by abalone, under static water flow conditions (Nakanishi 1978,

Jan and Chang 1983, Gaty and Wilson 1986, Nimura and Yamakawa 1989). Generally, oxygen uptake is directly related to metabolic rate and this relationship has been widely used to help indicate the health of animals and their overall energy expenditure or activity levels (Innes and Houlihan 1985). It is also a critical factor in assessment of stress in fishes (Beitinger and McAuley 1990).

As farming intensity increases, the DO regime and its impact on abalone growth will become more important to farmers. This study assesses the effects of chronic hypoxia on growth rate, and the effects that chronic hypoxia has on oxygen consumption of juvenile greenlip abalone, *H. laevigata*.

5.2 Materials and Methods

The juvenile greenlip abalone used in these experiments were approximately four years old. The initial mean length and wet weight of the abalone were 44.1±4.3 mm and 10.8±3.0 g (mean±SD; n = 603). Abalone used for this experiment were relaxed using aerated warm water (23-25°C) until they could be easily removed from tank surfaces. Subsequently, they were weighed, measured and tagged before being randomly distributed to 18 bioassay units.

5.2.1 Bioassay system

As each treatment was adjusted through the constant head chambers, the bioassay system was adapted to provide constant flow. Flow rates averaged 250.2±2.0 $ml.min^{-1}$ (n=72; 18 tanks on four occasions) giving an effective replacement rate of 90% of bioassay tank volume in 10-12 h. This was within the recommended flow rates for aquatic toxicological studies by Sprague (1969) of 90% replacement in 8-12 h. 200 and 300 W aquarium heaters in the bioassay tanks and constant head chambers, respectively, maintained relatively uniform daily temperature at 18.0±0.1°C (n=69 days) (range 17.1-19.4°C, except for a power failure on day 35 that resulted in a mean temperature of 14.9 °C for that day) (Table 5.1). The abalone were exposed to the treatments for up to 77 days.

The constant head chambers were also used for degassing, as described by Seidman and Lawrence (1985). As hypoxia experiments using nitrogen-stripped and vacuum-degassed test water produced similar mortality rates for three crustacean species (Nebeker et al. 1992), the more practical method of nitrogen stripping was employed to obtain the treatment levels. Nitrogen or oxygen gases were introduced to the constant head chambers via diffuser tubing to maintain the treatment levels. Gas flow was regulated individually for each constant head chamber.

5.2.2 Water quality analysis

The pH, temperature and DO in all tanks were measured on all days (Table 5.1). Nitrite was measured occasionally, and salinity was stable (34 ‰).

5.2.3 Experiment 5.1 : Chronic low dissolved oxygen exposure

One control and five experimental treatments were established (Table 5.1); average concentrations ranged from 8.9 - 4.2 mg $DO.l^{-1}$ The abalone were acclimatised to the bioassay system for 5-6 days before degassing commenced. All cages were checked daily for mortality. Food consumption was estimated on four occasions.

Tanks were also cleaned thoroughly, on average, every 15 days. This cleaning regime was less frequent than in previous bioassays (Chapters 3 and 4) and was adopted to reduce the likelihood of frequent changes in DO concentration. The DO level was measured in the buckets to determine if the cleaning process exposed the abalone to altered conditions. DO concentrations within the buckets did not differ by more than 11% from the tanks.

5.2.4 Experiment 5.2 : Oxygen consumption rates (post bioassay)

Commencing on day 57, abalone from the bioassay system were transferred to respirometer chambers for three days. These animals had been fed before removal. Temperature and pH levels were measured (Table 5.2).

5.2.5 Statistical analysis

The EC_5 and EC_{50} values, those concentrations where growth rate was reduced by 5 and 50%, respectively, were estimated from two intersecting linear regressions (Sedgwick 1979, Maguire and Hume 1982).

5.3 Results

5.3.1 Experiment 5.1 : Chronic low dissolved oxygen exposure

(SGR was significantly affected by hypoxia whether measured on a length ($p<0.01$) or whole weight ($p<0.001$) basis. For shell length, SGR declined with decreasing oxygen concentration over the entire experimental range (Figure 5.1). Shell growth relative to the control (7.7 mg $DO.l^{-1}$) was not significantly reduced ($p>0.05$) in oxygen treatments at 8.9 and 6.2-4.9 mg $DO.l^{-1}$ however, at 4.2 mg $DO.l^{-1}$, significant growth reductions occurred ($p<0.05$). Linear regression of the SGR data indicated that there was no shell growth below 4.3 mg $DO.l^{-1}$.

For WWBW gain, no significant growth reductions ($p>0.05$) occurred at oxygen levels of 8.9 and 5.6 mg $DO.l^{-1}$, however, significant growth reductions occurred at 6.2 and 4.9-4.2 mg $DO.l^{-1}$ (Figure 5.2) ($p<0.05$). Linear modelling of data for each replicate indicated an initial plateau in which growth was unaffected by DO in the range 8.9-7.7 mg $DO.l^{-1}$. The EC_5 and EC_{50} values from the weight data were 7.36 and 5.91 mg $DO.l^{-1}$, respectively.

DO depression had an effect on WWBW : SL, as this ratio declined between 8.92-5.55 mg $DO.l^{-1}$ (slope = 0.004), then declined more rapidly (slope = 0.027) as oxygen

decreased to 4.24 mg DO.l^{-1} (Figure 5.3). For treatments 5 and 6 (4.86-4.24 mg DO.l^{-1}) the ratios recorded were significantly different to the controls (p<0.05).

Survival was high in all but the two lowest DO concentrations (4.9-4.2 mg DO.l^{-1}), where significant reductions in survival occurred (p<0.05) (Table 5.1). Food consumption declined in a similar pattern (p<0.001), with significant depression of food consumption in treatments with 5.6-4.2 mg DO.l^{-1} (p<0.05) (Figure 5.4). There was a slight increase in pH values with decreasing DO level (p<0.001), and treatments 3-6 (mean pH of 8.03 at 6.2-4.2 mg DO.l^{-1}) were significantly different to the controls (mean pH of 7.96 at 8.9 mg DO.l^{-1}) (p<0.05) (Table 5.1).

5.3.2 Experiment 5.2 : Oxygen consumption rates (post bioassay)

Oxygen consumption by juvenile greenlip abalone was significantly affected by the prolonged exposure to low DO concentration (p<0.01) (Figure 5.5). Significant reductions in oxygen consumption occurred following prolonged exposure to 4.9-4.2 mg DO.l^{-1} (63-55% saturation) relative to the controls (p<0.05). The EC_5 and EC_{50} values (5 and 50% reductions in respiration rate) were 6.16 and 5.19 mg DO.l^{-1} (80 and 68% saturation), respectively. Oxygen consumption was estimated to be independent of exposure to external oxygen concentration in the range 8.9-6.3 mg DO.l^{-1}.

Table 5.1 Water quality parameters and survival of juvenile greenlip abalone, *Haliotis laevigata*, within the chronic hypoxia trial (Experiment 5.1)

Treatment[1,2]	% O_2 Saturation	Dissolved oxygen (mg DO.l^{-1})					pH	Survival %[4]	Temperature ^{O}C
		Mean±SE	Percentiles[3]		Range[3]				
		(n=3)	25%	75%					
1	117	8.9±0.05	8.2	9.5	5.3	16.3	7.96	94.5±3.2	18.0±0.1
2[5]	100	7.7±0.06	6.9	8.3	3.5	11.7	7.98	98.2±1.8	17.8±0.3
3	81	6.2±0.14	5.6	6.7	4	8.9	8.00	88.4±4.9	18.3±0.02
4	73	5.6±0.08	5.0	6.2	3.5	7.4	8.02	88.0±3.8	17.9±0.1
5	63	4.9±0.06	4.4	5.3	2.1	7.3	8.05	62.0±15.9*	17.8±0.1
6	55	4.2±0.02	3.7	4.6	2.4	6.9	8.06	59.2±9.6*	18.2±0.2

1. Ammonia concentrations were not significantly affected by treatment (p>0.05) (2-3±0.2-0.5 μg FAN.l^{-1}).

2. Nitrite concentrations were not significantly affected by treatment (p>0.05) (2-3±0.1-0.5 μg NO_2-N.l^{-1}).

3. Based on individual readings for bioassay units taken 3-4 times daily.

4. Mean values denoted by * were significantly different to the control (8.9 mg DO.l^{-1}) (p<0.05).

5. One of the triplicate tanks recorded 0.4 mg DO.l^{-1} due to a blocked inlet; this was rectified quickly and not included in this mean value.

Table 5.2 Experimental conditions for respirometry (Experiment 5.2).

Treatment[1]	Dissolved oxygen (mg DO.l^{-1})[2]	Day	Number of abalone	Total wet body weight (g)	Flow rate (ml.min^{-1})
1	8.9±0.05	74	23	294.6	124.8±2.2
2	7.7±0.06	71	25	292.0	127.2±9.1
3	6.2±0.14	68	24	272.4	151.2±14.3
4	5.6±0.08	63	23	276.5	116.8±17.5
5	4.9±0.06	60	21	218.1	147.2±9.1
6	4.2±0.02	57	21	214.4	150.7±4.7

[1] Average temperature and pH for all experimental trials did not differ significantly (p>0.05) and were 18.0±0.3°C and 7.99±0.02, respectively (n=5 trials).

[2] Treatment levels are nominal at concentrations sustained during chronic hypoxia exposure experiment

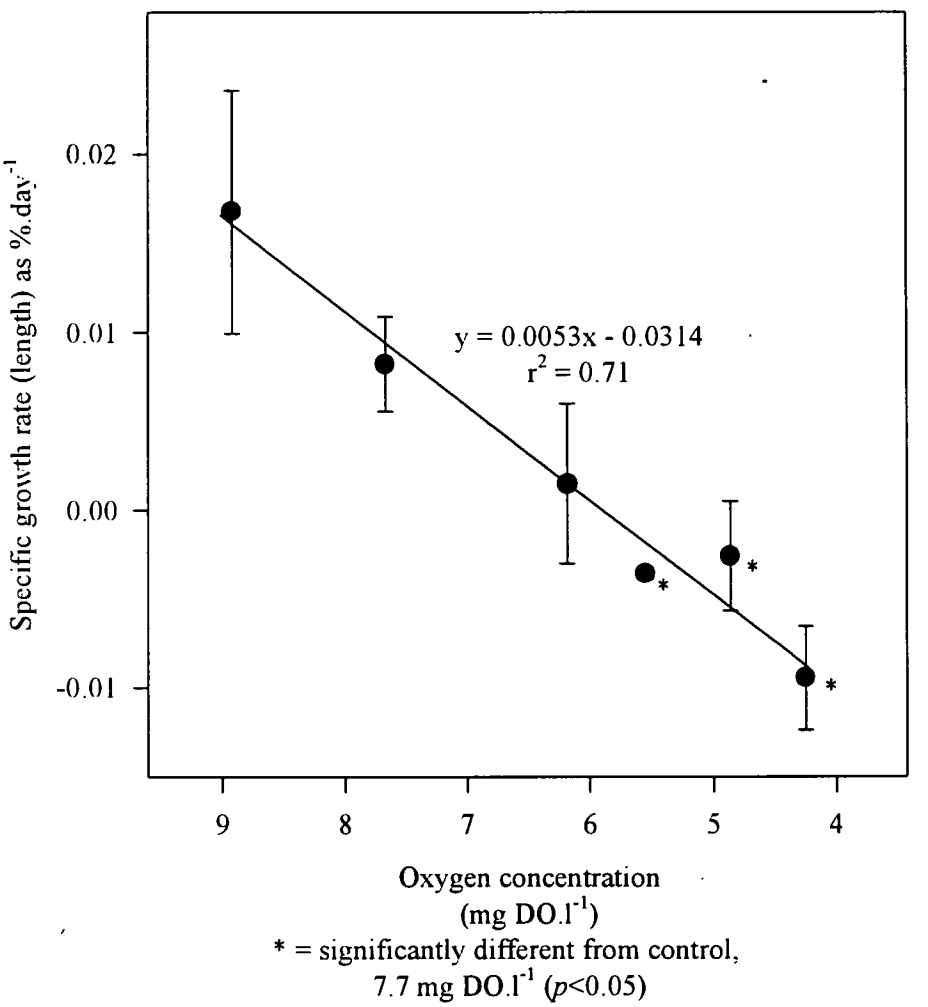

Figure 5.1 Specific growth rate (length) of juvenile greenlip abalone, *Haliotis laevigata*, subjected to chronic hypoxic conditions (mean±SE, n=3).

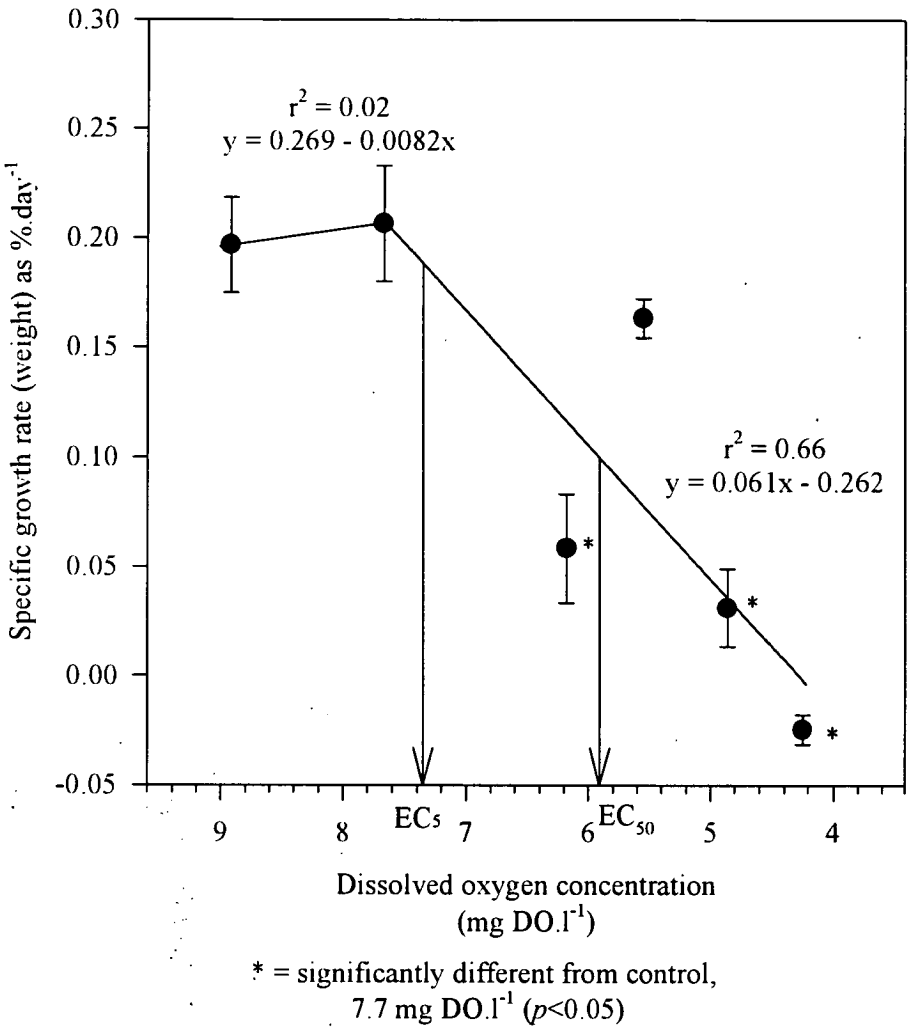

Figure 5.2 Specific growth rate (weight) of juvenile greenlip abalone, *Haliotis laevigata*, subjected to chronic hypoxic conditions (mean±SE, n=3).

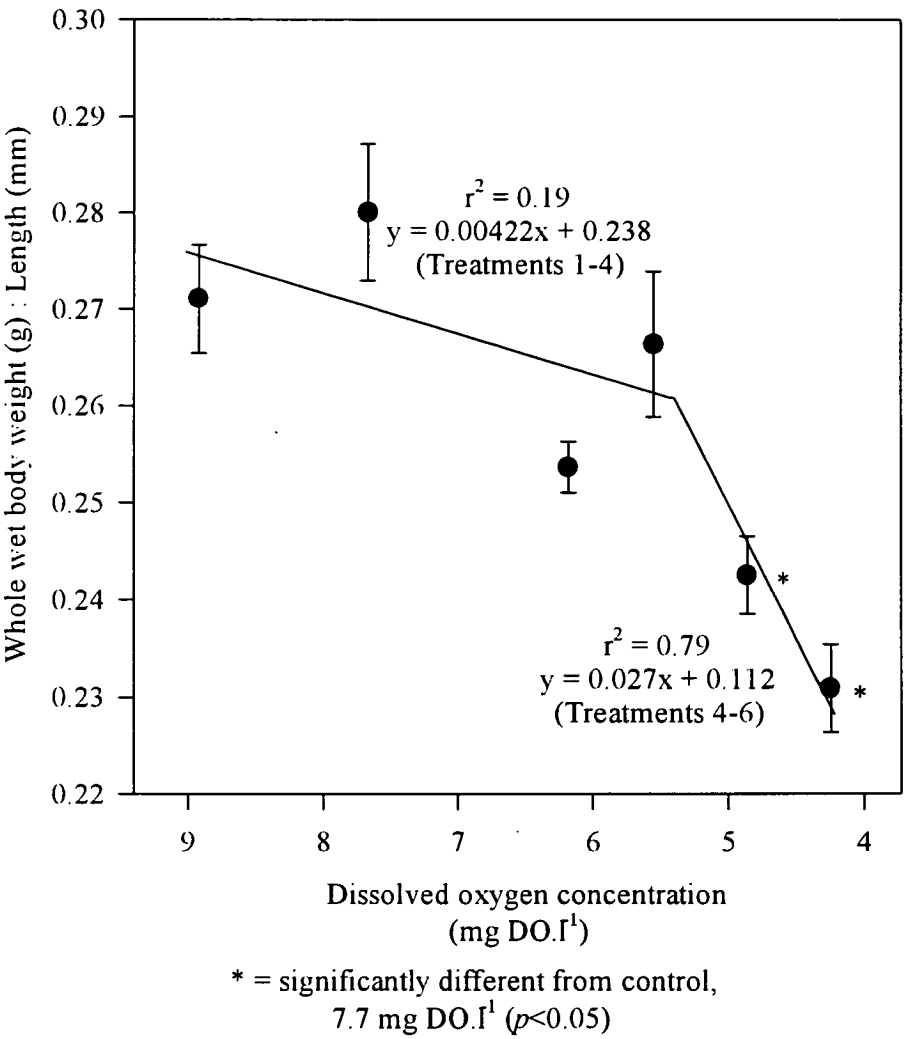

Figure 5.3 Whole wet body weight : shell length of juvenile greenlip abalone, *Haliotis laevigata*, subjected to chronic hypoxic conditions (mean±SE, n=3).

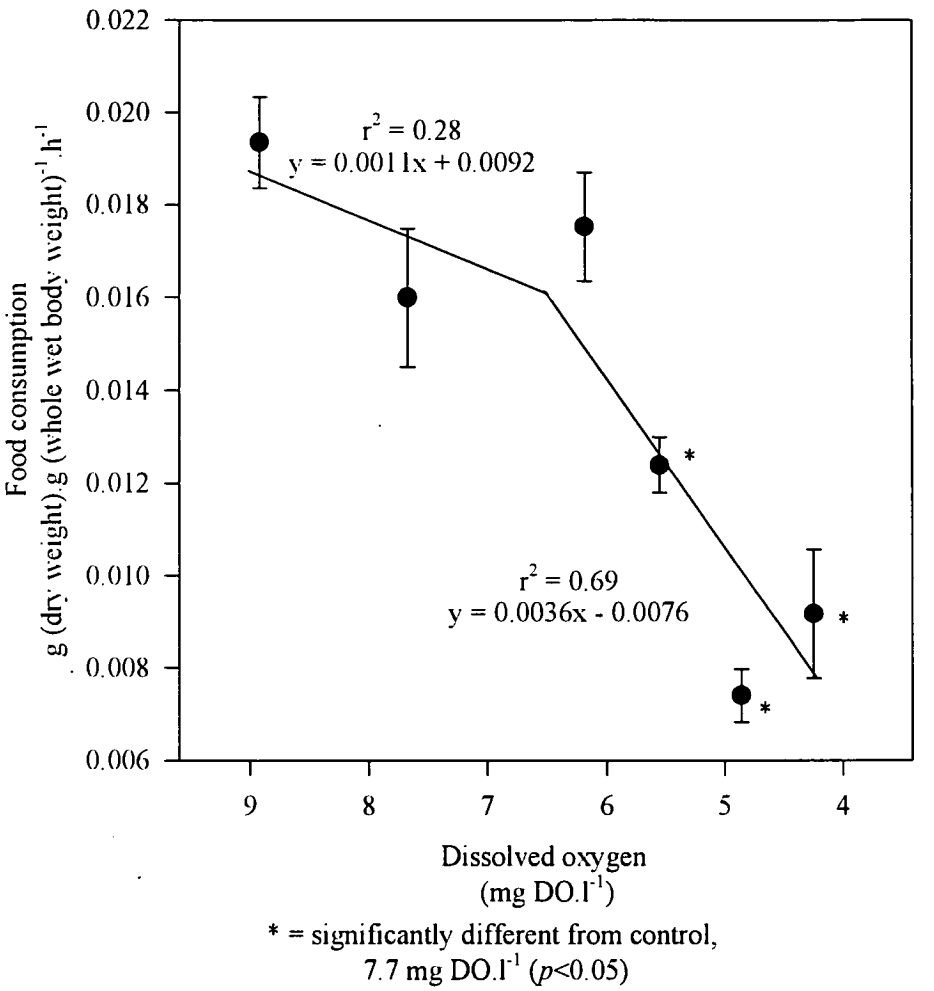

Figure 5.4 Food consumption of juvenile greenlip abalone, *Haliotis laevigata*, subjected to chronic hypoxic conditions (mean±SE, n=3), measured on four occasions.

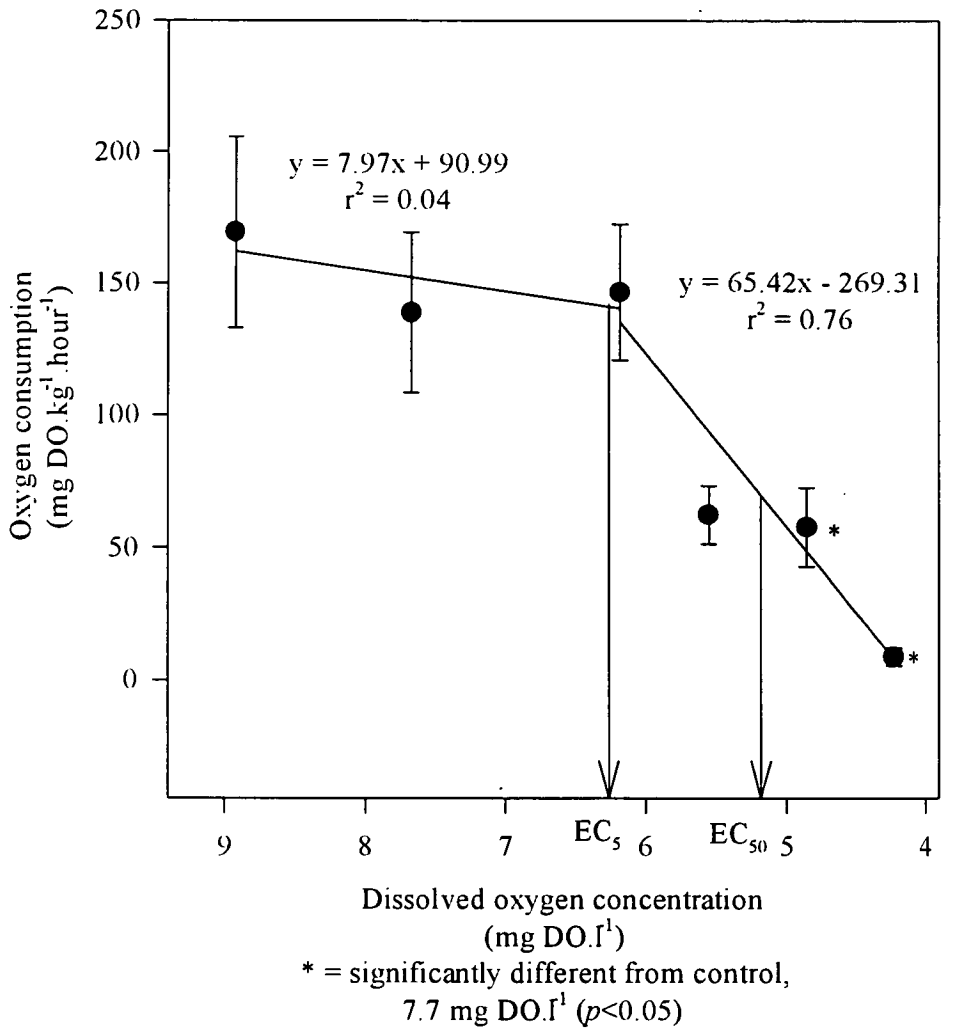

Figure 5.5 Oxygen consumption of juvenile greenlip abalone, *Haliotis laevigata*, subjected to reduced oxygen concentrations (mean±SE, n=3 except for 5.6 mg DO.l^{-1} where n=2).

5.4 Discussion

Growth rates of control animals (SGR weight = 0.21±0.03 %.day^{-1}; SGR length = 0.01±0.003 %.day^{-1}) were lower in comparison to a previous bioassay conducted at a similar temperature in the same experimental system (Chapter 4) (SGR weight = 0.48±0.04 %.day^{-1}; SGR length = 0.12±0.01 %.day^{-1}). An alteration in the method of aeration may explain this difference involved. In previous experiments (Chapters 3 and 4), aeration within the bioassay tanks may have contributed to greater water movement which is known to stimulate feeding (Shepherd 1973, Fleming et al. 1997). As the oxygen level within each tank was set externally in the current experiment, no water movement due to aeration occurred. Small submersible pumps were incorporated into the tanks to improve flow in the subsequent chronic bioassay (Chapter 6).

The lack of shell growth, especially at low DO levels, may be partly explained by the behaviour of the animals, as in treatments 5 and 6 (4.86-4.24 mg DO.l^{-1}) the abalone persistently attempted to escape the experimental conditions. Similar behaviour was observed by Nakanishi (1978) for *H. discus hannai* adults and by Jan and Chang (1983) for *Haliotis diversicolor supertexta* juveniles and adults when exposed to hypoxic conditions. Substantial shell-abrasion occurred in our study, as the animals interacted while attempting to escape. Regardless of the mechanism, the pattern is a clear response to experimental conditions. This is apparent from the whole wet body weight : shell length model (Figure 5.3), which declines rapidly below 5.5 mg DO.l^{-1}. DO appears to affect juvenile greenlip abalone, at low concentrations, more in terms of body weight, whereas shell growth is more sensitive than whole weight growth to moderate oxygen subsaturation. A similar trend in growth was evident in relation to ammonia concentrations during an earlier bioassay (Chapter 3).

The significant reduction in food consumption at 5.6 mg DO.l^{-1} (73% oxygen saturation) approximates the beginning of the region of significant growth rate depression (SGR weight). Thus reduced weight can be partly explained by the reduction in food consumption. Das and Stickle (1991) reported significant depression

of food consumption in the gastropod *Stramonita haemastoma* below 77% oxygen saturation. Beyers et al. (1994) found significant growth reductions over 11 months for the rock lobster *Jasus lalandii* at 35% oxygen saturation, with significantly depressed ingestion and increased mortality rates.

Dissolved oxygen concentrations at which sublethal effects were observed for several crustaceans, which use haemocyanin as the respiratory pigment as do abalone, are indicated in Table 5.4. Several fish species have also shown growth reductions in hypoxic conditions at 40-46% oxygen saturation (Brett and Blackburn 1981). The lower apparent tolerance of *H. laevigata* (significant reductions in food consumption, growth and survival at 63% saturation) suggests that greenlip abalone may be more sensitive to periods of hypoxia than several other aquatic animals. Similarly, food consumption data for *S. haemastoma* indicates that this may also apply to other gastropods (Das and Stickle 1991). The results of this study are consistent with those from a nitrite bioassay (Chapter 4), which showed that abalone were much more sensitive to nitrite, a respiratory poison, than several other aquatic animals. Abalone will tolerate short-term aerial exposure (Wells and Baldwin 1995) but clearly chronically hypoxic conditions are harmful.

The effect of external oxygen concentration on the oxygen consumption of abalone has been reported for two species of abalone, although these studies were conducted in static conditions. In their study of *H. diversicolor supertexta*, Jan and Chang (1983) demonstrated a relationship between oxygen consumption, wet body weight and the oxygen content of seawater. For abalone of 13 g WWBW, the decline in oxygen consumption with decreasing oxygen concentration (*Pc*) was apparent below 5.5 mg $DO.l^{-1}$, (80% oxygen saturation at 23°C), although no information was provided on feeding regimes. From Figure 5.5, the *Pc* value for greenlip abalone is 6.27 mg $DO.l^{-1}$, or 82% oxygen saturation. Gaty and Wilson (1986) suggest that the zone of respiratory independence for *Haliotis tuberculata* juveniles is extended to more hypoxic conditions with increasing age and in fed animals. No *Pc* values were provided by Gaty and Wilson (1986), although some depression of oxygen consumption for fed animals was evident at 63% oxygen saturation. The data for *H.*

laevigata indicate a significant reduction in oxygen consumption after chronic exposure to 4.9-4.2 mg $DO.l^{-1}$, or 63-55% oxygen saturation for a maximum of 77 days. The observations on respiration rate are consistent with observations by Nimura and Yamakawa (1989) of heart rate reductions for *Sulculus supertexta* below 55% oxygen saturation, and with Jan and Chang (1983) for the *Pc* value for abalone of similar size to those in our study. This decline in oxygen consumption rate occurred at levels well below where growth was affected, indicating the metabolic cost of oxygen regulation (Herreid 1980).

The slight increase in pH with decreasing DO treatment (Table 5.1) indicates that CO_2 buildup in the bioassay chambers was not a problem, and data for bivalves suggest that pH only affects growth when below 7 (Bamber 1990). The pH levels encountered in this experiment were within the EC_5 estimates for this species (Chapter 6). The levels of ammonia and nitrite were below that considered to be a confounding influence (Chapters 3 and 4). Although the treatments experienced some variation in oxygen levels (Table 5.1), all treatments were at significantly different concentrations ($p<0.001$). Previous work on hypoxia (Seidman and Lawrence 1985) demonstrated that daily fluctuation in DO concentrations was greater than in our study. Furthermore, the 25^{th} and 75^{th} percentiles (Table 5.1) indicate that our treatment levels maintained reasonable stability on a daily basis.

The results of this study may be applied to the management of abalone growout systems, however the conclusions drawn will be more robust when data are available for more rapidly growing individuals and for abalone experiencing combinations of stresses that are more likely in 'crises' in commercial aquaculture systems (Tomasso 1996). Future research in this experimental system should involve assessing effects of low DO in combination with elevated concentrations of nitrogenous wastes and should allow reassessment of tolerance to hypoxia for more rapidly growing abalone exposed to higher current speeds.

Table 5.4 Growth reductions observed in other aquatic animals which were subjected to hypoxic conditions and rely on haemocyanin.

Common name	Scientific name	Observations	Author(s)
Yabbies	*Cherax destructor*	Growth reductions <2.5 mg $DO.l^{-1}$ (<30% saturation)	Ingerson and Geddes 1995
Marine shrimp	*Penaeus semisulcatus*	Moulting inhibited at 2 ppm; significant mortality (27% saturation)	Clark 1986
	Penaeus vannamei	Significant growth reductions at 1-2 ppm (16-32% saturation)	Seidman and Lawrence 1985
	Penaeus monodon	Significant growth reductions at 1-2 ppm (16-32% saturation)	Seidman and Lawrence 1985
Rock lobster	*Jasus lalandii*	Significant mortality, growth reductions and reduced ingestion at 35% saturation	Beyers et al. 1994

6 EFFECT OF pH ON GROWTH RATE AND OXYGEN CONSUMPTION RATE FOR JUVENILE GREENLIP ABALONE.

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7 THE INFLUENCE OF DIETARY HISTORY ON ACUTE AMMONIA TOXICITY TO JUVENILE GREENLIP ABALONE.

7.1 Introduction

The toxicity of ammonia is influenced by several factors, including pH, dissolved oxygen, temperature, ionic concentration and other toxic chemicals (Russo 1985, Russo and Thurston 1991). The nutritional status of the test species has received little attention but appears to be important (Bengston et al. 1985), and is thought to account for much of the unexplained variation typical of many toxicity tests (Sprague 1990). It is also believed that diets higher in protein may offer more protection against toxicity (Sprague 1990), as body composition, physiological and biochemical functions may be affected by diet (Rand and Petrocelli 1985).

Recent research into artificial diets has resulted in improved growth rates for greenlip abalone (Hone and Maguire 1996). The requirements of the abalone for some specific ingredients has been assessed (Coote et al. 1996), however, artificial foods for abalone still appear to be lacking nutritional factors present in natural food (Dunstan et al. 1998). The processing of food for artificial diets may also contribute to compromising the nutritional value. Although dependent on the type of process, vitamin and amino acid losses through some heat treatments can be up to 75% (Fellows 1990).

This chapter assesses the acute toxicity of unionised ammonia and the influence of preceding nutritional history for juvenile greenlip abalone, *H. laevigata*, the most widely farmed abalone in Australia, and known to be quite sensitive to chronic exposure to ammonia (Chapter 3). This research was undertaken in response to assessments, made by abalone farmers, in which they attributed mortality to the interaction of nutritional and environmental stresses.

7.2 Materials and Methods

The juvenile greenlip abalone used in these experiments were approximately four years old. The initial mean length and weight of the abalone were 56.24±6.22 mm and 22.35±6.48 g (mean±SD; n =96). Abalone used for this experiment were relaxed using aerated warm water (23-25°C) until they could be easily removed from tank

surfaces (Gilroy and Edwards 1998). Subsequently, they were weighed, measured, tagged (Hallprint, Adelaide, Australia) and randomly distributed to 16 bioassay units.

For 4 months prior to experimentation, these abalone were maintained on a mixture of three proprietary formulated abalone feeds (ABCHOW, Deakin, Promak). Half of the randomly assigned groups were assigned either the heat-treated food, or the normal food. The heat-treated food was the same mixture of three feeds as the normal group, but it was placed in the oven at 100 °C for two days. The diets were analysed by Allison Laboratories, Hobart in order to determine available protein. Pepsin digestibility, as per Olley and Pirie (1966), of the normal food was 79.6%, while for the heat-treated diet it was 79.8%. Water soluble protein of the normal diet was 7.4%, whereas for the heat-treated diet it was 4.8% (Appendix 3).

7.2.1 Bioassay system

In each 70 l bioassay tank, there was one cage suspended vertically, containing 12 abalone. Daily flow rates averaged 159.9±1.3 $ml.min^{-1}$ (n=3) giving an effective replacement rate of 90% of bioassay tank volume in 12 hours. This was within the recommended flow rates for aquatic toxicological studies by Sprague (1969) of 90% replacement in 8-12 hours. The experiment was conducted using 200-300 W aquarium heaters in the bioassay tanks and head adjustment columns, respectively, to maintain relatively uniform daily temperature at 19.6±0.2°C (n=5) (range 19.3-20.5 °C) (Table 7.1). Relatively uniform pH levels were maintained across all treatments (8.10) although treatment 4 was significantly lower than the control (p<0.05) at 8.06. The effect of this on the ionisation of ammonia was low, as the percentage of FAN decreased by from 3.73% to 3.28% (Bower and Bidwell 1978). A valve in the base of each bioassay tank was opened daily to remove organic wastes, but the tanks were not cleaned during the acute bioassay.

7.2.2 Water quality analysis

The pH and temperature in all tanks were measured on all days, and ammonia measured on four occasions (Table 7.1).

7.2.3 Acute toxicity of ammonia

One control and three experimental ammonia treatments were established (Table 7.1); average ammonia concentrations ranged from 0.003 - 1.025 mg FAN.l^{-1} (0.084-30.244 mg TAN.l^{-1}). The treatments were also divided into two dietary combinations; either the normal food, or the heat-treated diet. This produced eight treatments in duplicate. The abalone were not acclimatised to the bioassay system before the addition of NH_4Cl as the concentrations were established prior to adding the animals. Aeration was provided and abalone were not fed during the bioassay. Individuals on the base of the cages that did not respond to touching were determined to be dead, and all dead abalone were removed. The numbers of dead abalone were recorded after 0.5, 1, 2 and at four hour intervals thereafter to 130 hours.

Table 7.1 Experimental conditions during acute ammonia exposure (mean±SE, n=4-5)

Treatment	Ammonia concentrations (mg.l^{-1})		pH	Temperature °C
	FAN	TAN		
1	0.003±0.0003	0.084±0.006	8.12±0.01	19.7±0.01
2	0.384±0.031	10.194±0.865	8.11±0.003	19.6±0.07
3	0.684±0.054	18.126±1.548	8.11±0.005	19.7±0.06
4	1.025±0.033	30.244±1.070	8.06±0.007	19.5±0.04

7.2.4 Statistical analysis

The median lethal time (LT50) value, or the time required to kill 50% of a population, was calculated using probit analysis (Sprague 1969). The effect of diet, ammonia concentration and their interaction were examined using Chi-squared (X^2) analysis (Sokal and Rohlf 1995).

7.3 Results

7.3.1 Acute toxicity of ammonia

No abalone died in the control tanks or treatment 2 (0.384±0.031 mg FAN.l^{-1}). At the higher FAN levels (0.684±0.054 and 1.025±0.033 mg FAN.l^{-1}) higher mortality rates were recorded (6.25 and 66.67% after 130 hours, respectively). Cumulative mortality data are shown in Figure 7.1. Chi-squared analysis indicated no significant effect of diet on mortality (p>0.05; 1 tailed, v=1, α= 0.05, X^2 calc. = 0.1875) but ammonia concentrations significantly affected mortality (p<0.001; 1 tailed, v=3, α= 0.001, X^2 calc. = 83.06). A chi-squared contingency analysis indicated that the effects of diet and ammonia concentration were independent (p>0.05; 1 tailed, v=1, α= 0.05, X^2 calc. = 0.4453), so diet replicates for treatment 4 (1.025±0.033 mg FAN.l^{-1}) were pooled in order to estimate the LT50. The probit of cumulative percent mortality for *H. laevigata* juveniles exposed to ammonia was linearly related to log(time) for treatment 4 (1.025±0.033 mg FAN.l^{-1}) (Figure 7.2). From this data at 1.025 mg FAN.l^{-1}, an LT50 value of 125.3 h was estimated.

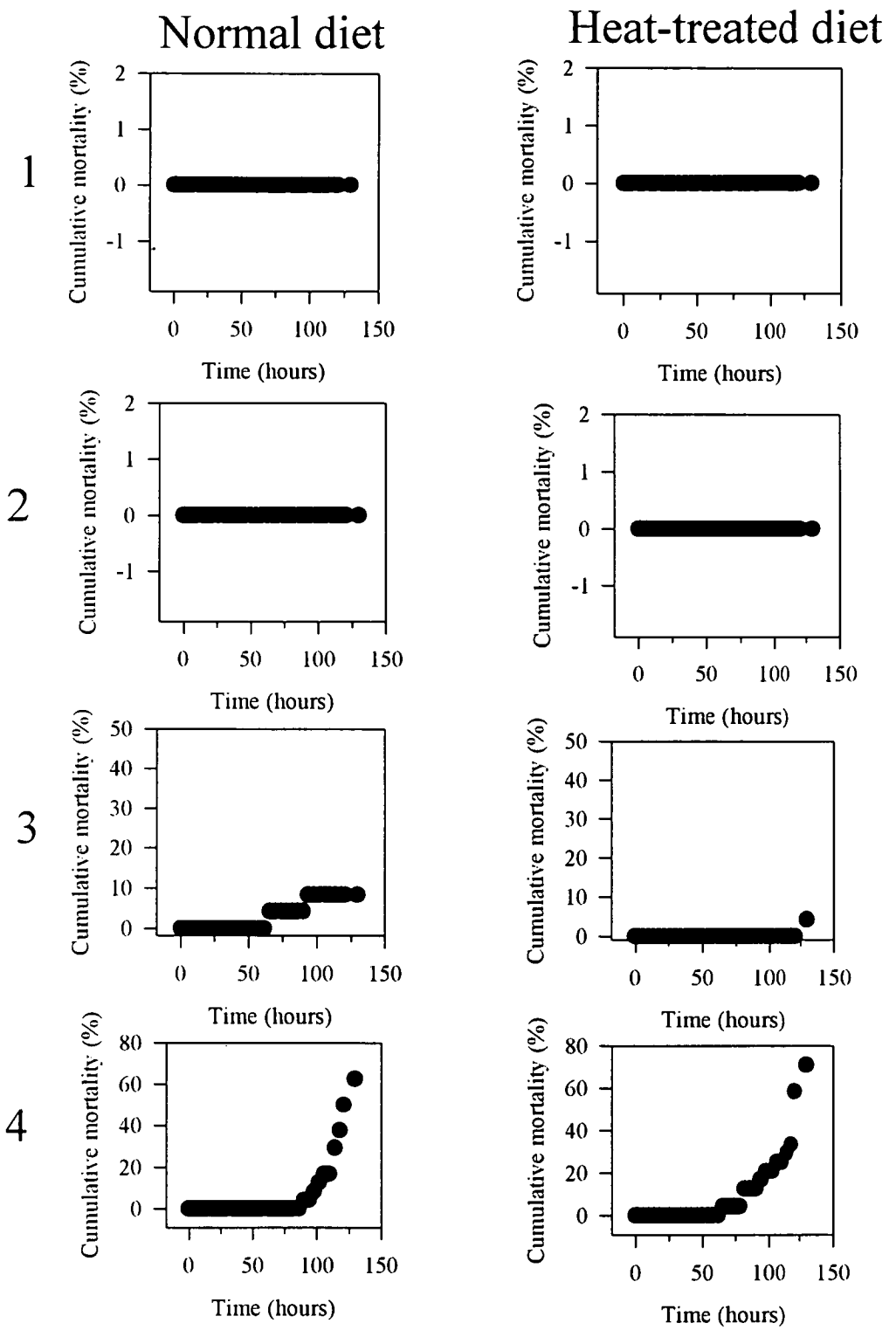

Figure 7.1 Cumulative mortality graphs for all (2 diets x 4 ammonia concentrations = 8) treatments in an acute ammonia exposure experiment with juvenile greenlip abalone, *Haliotis laevigata* (concentrations given in Table 7.1).

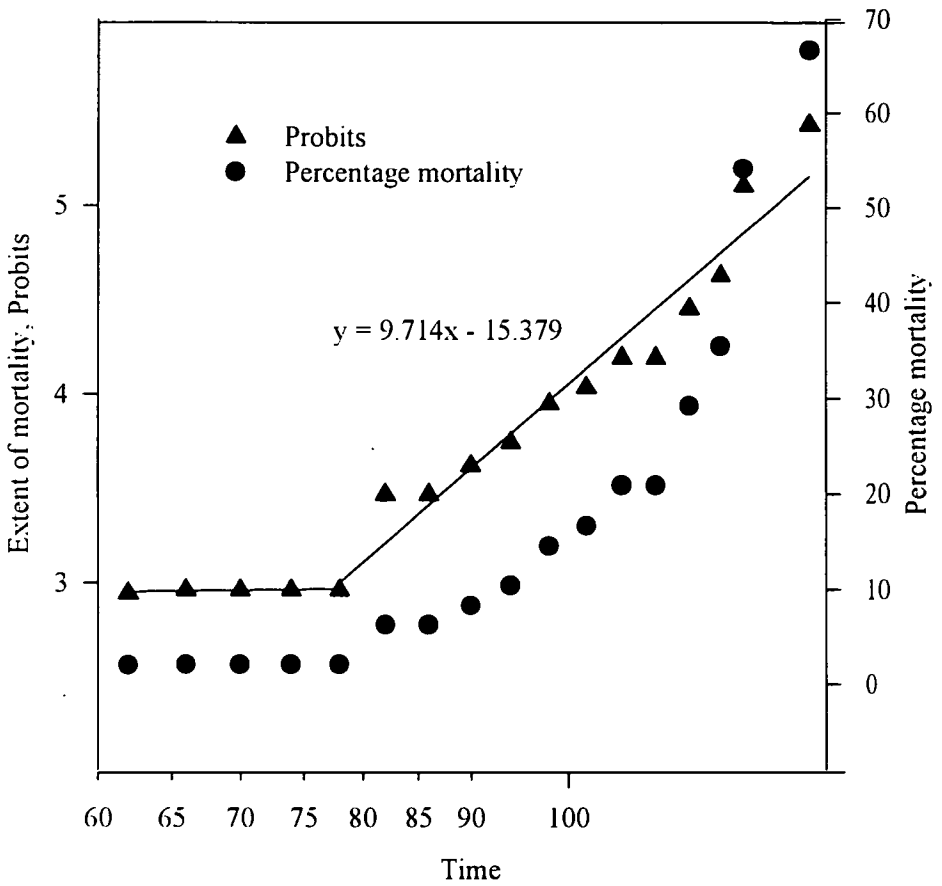

Figure 7.2 The cumulative mortality and probit analysis of mortality against log (time) at 1.025±0.033 mg FAN.l^{-1} for juvenile greenlip abalone, *Haliotis laevigata*, in an acute bioassay (data for two dietary treatments pooled) (▲= probits, ● = cumulative percent mortality).

7.4 Discussion

The preceding nutritional history of the juvenile greenlip abalone had no appreciable effect upon survival when the abalone were exposed to high ammonia concentrations. In a review of the effects of nutrition on response to toxicants, Bengston et al. (1985) concluded that the sensitivity of fish to organic pollutants and heavy metals increases

with declining protein and amino acid levels, vitamin C, and increasing carbohydrate in the diet. Winner et al. (1977) determined differences in the acute and chronic response to copper of *Daphnia magna* maintained on two diets; one was vitamin enriched. They found no difference in the response of either diet group to acute copper toxicities, yet diet significantly affected the chronic response. Winner et al. (1977) suggested that differing toxic mechanisms are at work for the nutritional influence on acute and chronic toxicity.

Maguire et al. (1996b) demonstrated that the heat treatment used for this experiment, when applied to one of the diets used in this study (ABCHOW), depressed growth of juvenile greenlip abalone by 43.4%. Although the diets in this study did not differ in their pepsin digestibility values, the difference in the water soluble protein content indicated that alterations occurred within the protein content. However, whether these actually translated into the desired detrimental effect on the abalone is less evident.

Recent research has shown that the vitamin content of an abalone diet can influence the tolerance to low salinity (Boarder and Maguire 1998). Vitamin losses through heat treatment can be up to 75% for thiamine and 35% for pantothenic acid, although substantial variation may occur due to the type of food and the method of preparation (Fellows 1990). It may be that tolerance to acute ammonia levels is less critically mediated by vitamins as cofactors, than acute tolerance to low salinity. Conversely, the ABCHOW diet has recently been shown to have only half of the sufficient vitamin levels required for greenlip abalone (Boarder and Maguire 1998), limiting the likelihood of nutritional differences.

It appears that juvenile greenlip abalone differ little from other marine species in their acute sensitivity to ammonia (Table 7.2), with bivalves being the notable exception due to their ability to close their valves (Epifanio and Srna 1975). In contrast, greenlip abalone exhibit a high degree of sensitivity to ammonia, compared to many other species, when subjected to chronic exposure (Chapter 3). This relatively high tolerance may be helpful in enduring short-term crisis events in commercial culture systems.

Table 7.2 The sensitivity of some marine invertebrates to acute ammonia toxicity

Common name	Species name	Ammonia level ($mg.l^{-1}$)		Mortality	Author(s)
		FAN	TAN		
American oyster	*Crassostrea virginica*	4.6-34.3	154-1150	96h lethal tolerance limit	Epifanio and Srna 1975
Hard clam	*Mercenaria mercenaria*	3.3-6		96h lethal tolerance limit	Epifanio and Srna 1975
Marine shrimp	*Penaeus monodon* (adolescents)	0.96	53.4	96 h LC50	Chen et al. 1990a
	P. monodon (juveniles)	1.08	38.00	120 h LC50	Chen and Lei 1990
	Penaeus chinensis (juveniles)	1.44	35.09	120 h LC50	Chen et al. 1990b
	Penaeus japonicus (juveniles)	3.00	40.31	96 h LC50	Kou and Chen 1991
Greenlip abalone	*Haliotis laevigata*	1.025	30.24	125 h LT50	This study

8 HISTOPATHOLOGY OF GILL AND KIDNEY TISSUE.

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Effects of chronic exposure of greenlip abalone, Haliotis laevigata Donovan, to high ammonia, nitrite and low dissolved oxygen concentrations on gill and kidney structure

Chapter 8 has been removed for copyright or proprietary reasons.

9 IONIC COMPOSITION OF HAEMOLYMPH.

9.1 Introduction

Exposure to toxicants can affect aquatic organisms in several ways. The overt signs of toxicity are nearly always preceded by biochemical, physiological and/or morphological changes in the organism (Meyers and Hendricks 1985), which can in turn affect such aspects as growth, reproduction and survival. Many toxicants interfere with osmoregulatory ability (Wickins 1981) and hence alter internal osmotic concentration (Chen and Chen 1996). The haemolymph of marine molluscs constitutes 30-80% of soft body parts and is very like the surrounding seawater in ionic composition, although marine molluscs often have slightly hyperosmotic haemolymph (Burton 1983). Haemocyanin is the main respiratory pigment in gastropods such as abalone. The centre of the oxygenating ability of this pigment molecule is the presence of copper. In other species, all copper within the haemolymph has been observed associated with haemocyanin (Djangmah and Grove 1970), and has been used previously for indirect measurements of respiratory pigment level (Prosser and Brown 1961). In contrast, organic osmolytes such as free amino acids are utilised by marine molluscs for intracellular ionic regulation (Somero and Bowlus 1983) and can also be measured to determine the physiological state of the organism (Bellchambers 1998) As haemolymph ionic levels are more likely to be influenced by altered external ionic conditions than the organic osmolytes, they hold some promise for determining the effects of these altered conditions (Boarder 1997).

Abalone possess the respiratory pigment haemocyanin (Ainslie 1980a), which is also known to have altered affinity for oxygen when ionic strength changes (Brix 1983). The purpose of this study was to assess the effects of sublethal chronic exposure of juvenile greenlip abalone, *H. laevigata*, for 2-3 months, to high ammonia and nitrite levels, low dissolved oxygen and extreme pH on ionic levels within the haemolymph. This research also complements previous work on histopathological changes associated with exposure to these toxicants during bioassays (Chapter 9) and effects on growth (Chapters 3,4,5 and 6). This research is part of a search for physiological indicators of sufficient stress to affect growth.

9.2 Materials and Methods

9.2.1 Abalone

Juvenile greenlip abalone were sampled after chronic sublethal exposure for 58-82 days to ammonia (as NH_4Cl) (Chapter 3), nitrite (as $NaNO_2$) (Chapter 4), low dissolved oxygen (Chapter 5) and a range of pH conditions (Chapter 6). The experimental ranges were 0.006-0.188 mg FAN.l^{-1} (0.237-9.04 mg TAN.l^{-1}), 0.024-7.80 mg NO_2-N.l^{-1} and 8.9-4.2 mg DO.l^{-1} (117-57% DO saturation) and pH 9.01-6.79 (Table 9.1).

9.2.2 Haemolymph sampling

Five abalone were sampled from two of the triplicate bioassay tanks for each treatment, weighed to the nearest 0.01 g and measured with callipers to 0.1 mm (Table 9.1). A small cube of tissue was removed from the centre of the ventral foot surface, and haemolymph from the pedal sinus was allowed to collect for 2-3 minutes. This site is known to be effective for obtaining haemolymph samples from abalone (Chen 1996). Clean pasteur pipettes were used for each sample. Haemolymph samples (<1 ml) were immediately centrifuged for several minutes to remove haemocytes (Voltzow 1994), before the haemolymph was removed, immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

9.2.3 Haemolymph analysis

The tubes were thawed immediately prior to analyses. Measurement of all ions except copper took place on a Roche Cobas-MIRA automatic chemical analyser. In order to determine haemolymph sodium, potassium, chloride and calcium, the haemolymph needed to be diluted 1:4, and for magnesium, a further dilution to 1:40 was required. Calcium and magnesium levels were only measured on samples from the pH bioassay (Chapter 6). Both sodium and potassium were directly measured using ion-selective electrodes. Chloride ion concentration was determined spectrophotometrically using

the thiocyanate method (Cobas-MIRA 1987). Calcium ion concentration was determined spectrophotometrically using the arsenazo III method, while magnesium was similarly determined using the arsenazo method (Cobas-MIRA 1987). Copper concentration was determined on a Varian Atomic Absorption Spectrometer 300+, by adding 0.1 ml of haemolymph to 1 ml of 20% trichloroacetic acid and reading at 324.8 nm.

9.2.4 Statistical analysis

The data for calcium concentration from haemolymph samples obtained during the pH bioassay displayed a high degree of variability for pH 9.01. After transformation, variances were still heterogeneous. Consequently, this level was omitted during statistical analysis.

9.3 Results

Ionic composition of seawater in control groups not exposed to atypical concentrations of ammonia, nitrite, dissolved oxygen and pH resembled normal seawater composition (Burton 1983) (Table 9.1). Exposure to chronic, sublethal ammonia concentrations resulted in no significant differences ($p>0.05$) to haemolymph potassium, chloride or copper levels, however, haemolymph sodium was significantly affected by external ammonia concentration ($p<0.01$). Depression of haemolymph sodium occurred at 0.110±0.009 mg FAN.l^{-1} ($p<0.05$), but not at 0.188±0.016 mg FAN.l^{-1} (Figure 9.1).

Exposure to chronic, sublethal nitrite concentrations resulted in no significant differences ($p>0.05$) in haemolymph sodium, potassium or copper levels, however, haemolymph chloride was significantly affected by external nitrite concentration ($p<0.05$). Depression of haemolymph chloride occurred at 7.80±0.23 mg NO_2-N.l^{-1} ($p<0.05$) (Figure 9.2).

Exposure to chronic, sublethal dissolved oxygen concentrations resulted in no significant differences ($p>0.05$) to haemolymph sodium, potassium, chloride or copper levels.

Chronic exposure to a range of pH conditions resulted in no significant differences in haemolymph sodium, potassium, chloride, copper and magnesium levels. A significant difference was noted in calcium levels ($P<0.01$), with increasing haemolymph calcium with increasing acidity occurring (Figure 9.3). After the data for pH 9.01 were omitted due to high variability, significantly higher haemolymph calcium concentrations were observed in abalone from pH 7.76-7.16 as compared to the controls.

Table 9.1 Weights, lengths and values of haemolymph solute concentration for all abalone.

Toxicant	Treatment	Length (mm)	Weight (g)	n	Na^+	Cl^-	K^+	$Na^+:K^+$	Cu^{2+}	Ca^{2+}	Mg^{2+}
pH	Control	36.0±3.5	5.62±1.76	10	477±7	520±2	17.1±0.7	28±1	295±13	9.0±0.6	46.6±1.2
	Others	31.5±2.6	3.82±1.08	43	485±10	527±5	17.1±0.4	29±1	233±18	10.3±0.2	45.2±1.5
Ammonia	Control	36.1±5.2	6.75±3.01	15	445±7	475±32	13.6±1.0	33±2	214±42	-	-
	Others	34.4±4.7	5.78±2.49	55	443±8	494±14	14.2±0.6	32±2	194±37	-	-
Nitrite	Control	39.9±3.1	9.13±2.23	10	463±1	510±7	13.5±0.5	35±1	140±30	-	-
	Others	37.9±3.4	8.04±2.27	50	453±3	498±7	13.0±0.3	35±1	121±9	-	-
Dissolved oxygen	Control	43.6±4.4	12.26±3.63	10	465±8	527±10	12.4±0.6	38±1	95±23	-	-
	Others	45.4±3.9	13.15±3.68	50	464±3	523±4	12.4±0.3	38±1	69±4	-	-

n= Total number of replicates drawn from 18 tanks for each of six experimental toxicant concentrations

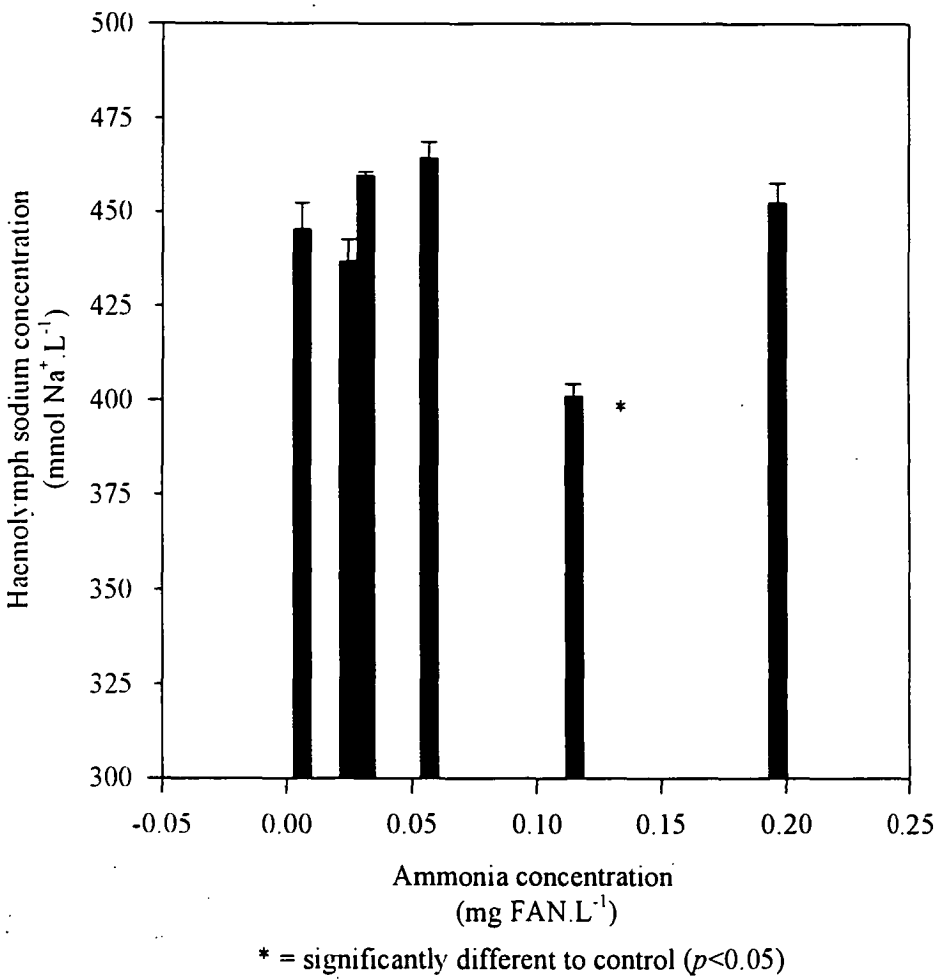

Figure 9.1 Haemolymph sodium concentration in juvenile greenlip abalone, *Haliotis laevigata*, subjected to chronic ammonia exposure (mean±SE).

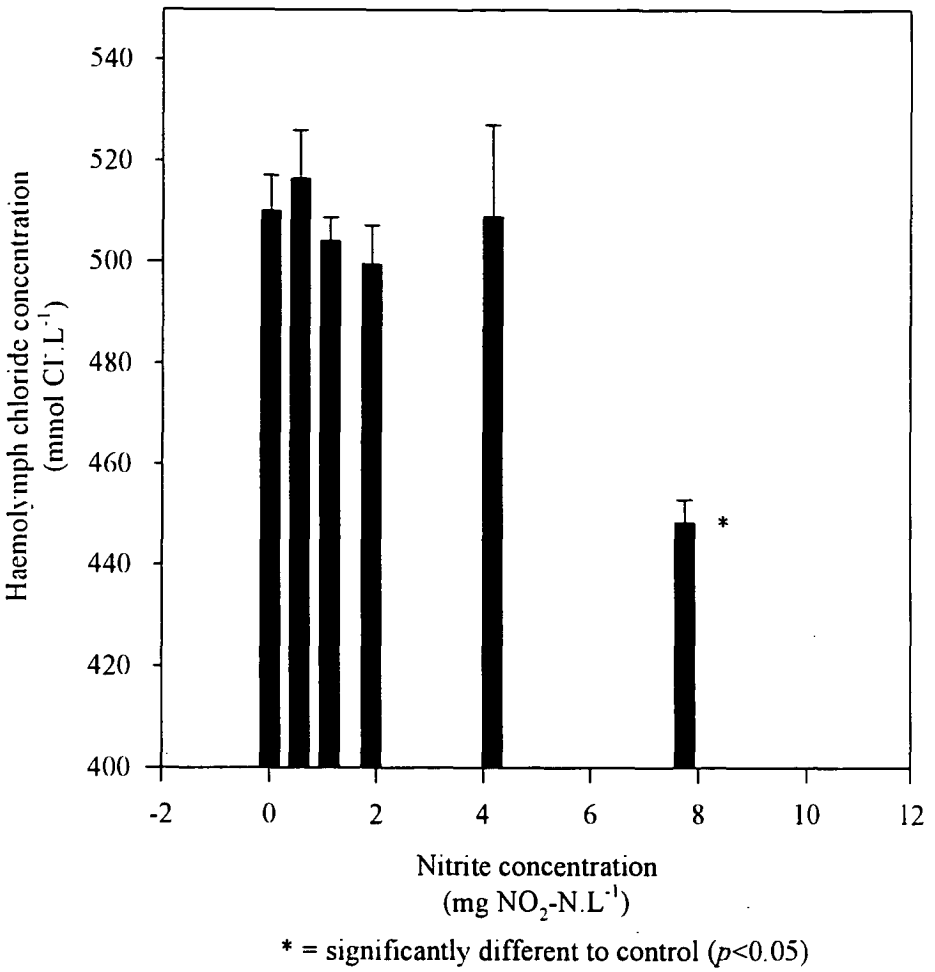

Figure 9.2 Haemolymph chloride concentration in juvenile greenlip abalone, *Haliotis laevigata*, subjected to chronic nitrite exposure (mean±SE).

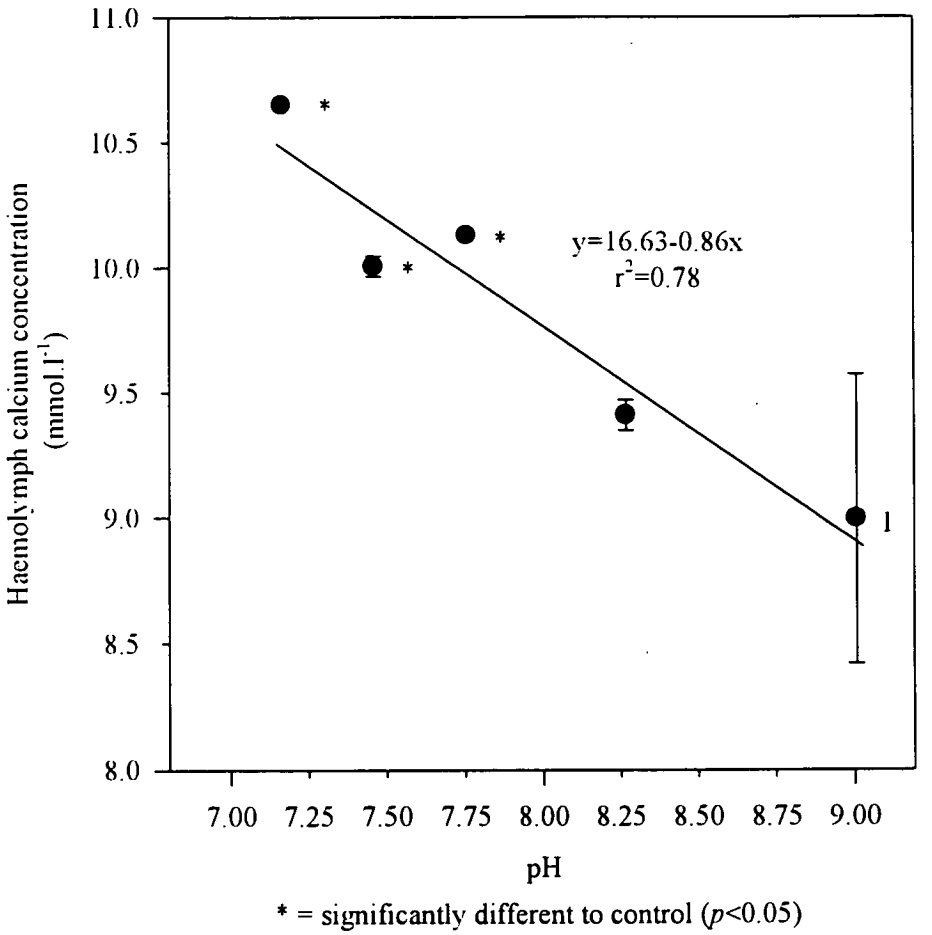

Figure 9.3 Haemolymph calcium concentration in juvenile greenlip abalone, *Haliotis laevigata*, exposed to a range of pH conditions (mean±SE). Regression based on individual tank values rather than treatment means (n=3). 1. This treatment was excluded from statistical analysis due to high variability.

9.4 Discussion

Limited information is available on the effects of chronic exposure to similar toxicants on the ionic chemistry of aquatic invertebrates. However, several acute toxicity studies have demonstrated similar responses to those of greenlip abalone. Acute

exposure to ammonia (32 mg TAN.l^{-1}) produced similar depression of haemolymph sodium levels for post-larval American lobster, *Homarus americanus* (Young-Lai et al. 1991). Similarly, acute exposure of *P. japonicus* to ammonia (20.7 mg TAN.l^{-1}) resulted in depressed haemolymph sodium, calcium, magnesium and chloride ion concentrations (Chen and Chen 1996).

For aquatic animals in conditions of high external ammonia, a net inward diffusion of ammonia occurs, possibly balanced via Na^+/NH_4^+ and/or Na^+/H^+ exchange (Cameron and Heisler 1983). If a marine gastropod such as abalone has similar Na^+/NH_4^+ exchange mechanisms to other molluscs (McCorckle and Dietz 1980), then most inwardly diffusing NH_3 would be converted to NH_4^+ at haemolymph pH (Thurston and Russo 1981). As internal solute concentration rose, NH_4^+ would be transported out of the haemolymph via Na^+/NH_4^+ exchange, which should result in increased internal sodium concentrations. However, the role of Na^+/NH_4^+ exchange is still in question (Evans and Cameron 1986), as Lucu et al. (1989) reported very little NH_4^+ exchange in the crab *Carcinus maenas* as a means of removal. The excretion of ammonia in another gastropod mollusc, *Busycon canaliculatum*, occurs with the aid of Na^+K^+-ATPase (Mangum et al. 1978).

Survival of abalone at ammonia treatment 5 (0.110 mg FAN.l^{-1}) was 95% over the duration of the trial (Chapter 3), however abalone of this treatment demonstrated an effect on haemolymph sodium. Survival was depressed at treatment 6 (0.188 mg FAN.l^{-1}) and no effect was observed. This situation may represent the compensation of Na^+/NH_4^+ exchange at sublethal levels that are compromised at higher concentrations, however, more research is necessary in order to validate this.

The depletion of haemolymph chloride levels from abalone exposed to nitrite is a characteristic effect in nitrite-exposed fish and crustaceans (Jensen 1996). The active branchial uptake of Cl^- is converted partially to NO_2^- uptake, while passive efflux of Cl^- occurs, resulting in a net loss of chloride with increasing external nitrite. In carp, a linear relationship exists between plasma chloride and the sum of nitrite, lactate and bicarbonate concentrations, indicating that HCO_3^-/ Cl^- exchange is also removing

plasma chloride in this species (Jensen et al. 1987). However, Jeberg and Jensen (1994) demonstrated that for the freshwater crayfish, *Astacus astacus*, nitrate must be involved in the regulation of nitrite as well as chloride. The depression of haemolymph chloride ions in response to exposure to nitrite at 7.80 mg NO_2-N.l^{-1} did not correlate with survival, as abalone from this treatment recorded survival not significantly different to control abalone (Chapter 4).

The pattern of increased calcium concentration in greenlip abalone haemolymph with decreasing pH is known to occur for other molluscs. The decrease in pH is believed to cause dissolution of $CaCO_3$ from the shell, and the subsequent rise in calcium in the extrapallial fluid is countered by a rise in haemolymph calcium levels (Burton 1983). A localised decline in pH is usually associated with anaerobic respiration, and an accumulation of organic metabolites in molluscs (Burton 1983), including abalone (Wells and Baldwin 1995). Visible erosion of abalone shells that occurred in the acidic treatments (Chapter 6) supports this view of shell dissolution influencing haemolymph calcium levels.

The variation in copper ions observed in control abalone between the different experiments (Table 9.1) is indicative of southern Australian abalone, which have demonstrated variation in haemocyanin concentration not corresponding with season, water temperature, feeding behaviour or reproductive state within a species (Ainslie 1980b). The data presented here suggest that haemolymph copper levels may decline with age, however, much more work is required to validate this in view of the variation in copper levels experienced in this study and by previous authors (Ainslie 1980b). Although it was hoped that some response to toxicants might occur in haemolymph copper levels, no changes were noted and it appears unlikely as a useful indicator of metabolic stress. Although no changes were noted in these experiments, potassium levels are known to be regulated in marine gastropods (Burton 1983) including greenlip abalone (Boarder 1997).

Current attempts at determining stress indicators in abalone have produced some useful measures (Boarder 1997, Chapter 8). The use of ionic composition of

haemolymph, however, seems limited in assessing sublethal stress, as significant effects on ionic composition appear only at levels where survival is also reduced, or are inconsistent with increasing toxicant level (eg FAN).

10 GENERAL DISCUSSION

The experiments on chronic toxicity began with exposure to high ammonia, then high nitrite, followed by low dissolved oxygen and a range of pH conditions. The initial ammonia experiment was conducted at ambient temperature and was of longer duration than succeeding bioassays. In an attempt to increase growth rates, and hence reduce experimental duration, immersion heaters were used for the nitrite and succeeding bioassays. The control abalone in the experiment on nitrite exposure demonstrated an increase in growth over the control abalone from the previous, ammonia bioassay, at least in terms of WWBW gain (Table 10.1). The third bioassay, on chronic exposure to a range of dissolved oxygen levels, could not be conducted with aeration in the bioassay tanks as in the previous two bioassays, as this would have impacted on treatment levels. Even with the use of heaters, control abalone of this experiment demonstrated reduced growth rates, especially in terms of length (Table 10.1). Due to this reduction in growth, submersible pumps were used in the subsequent bioassay on pH. The substantial increase in growth rates of control abalone within the chronic pH bioassay indicated that this strategy worked, and also anecdotally supports recent research into the effect of water velocity on greenlip abalone (Fleming et al. 1997, Higham et al. 1998).

Within Australia, abalone are increasingly being grown in tanks on land (Hone and Maguire 1996), where conditions may vary from the relatively stable conditions experienced by abalone in wild situations. Situations where water is re-used to some extent allow the development of nitrifying bacterial populations (Wickins 1983) that can, in turn, affect water quality. These bacteria will oxidise ammonia to nitrite, then into nitrate, while acidifying the surrounding environment and placing demands on the oxygen content of the water (Wickins 1983). Thus, a normally operating biofilter will cause ammonia levels to decrease, while nitrite levels increase until sufficient bacteria develop to convert the nitrite into nitrate, while decreasing pH and dissolved oxygen (Jirsa et al. 1997). The implications for water re-use depend on the nitrogen loading of the water and the processing capacity of the biofilter (Colt and Armstrong 1981), although the overall effect is of several aspects of water quality altering, over time, to levels that abalone may not experience in the wild.

As a result of this research, recommended levels for pH, DO, NH_3 and NO_2 for abalone culture have been made. Each aspect of water quality, when at levels far from optimal, produced growth reductions. As the estimates for individual parameters, they hold true when the impact of a single aspect of water quality is assessed. However, in situations where water re-use occurs, the likelihood is that each of these aspects may vary to sub-optimal levels at the same time and possibly produce different responses to those observed for individual toxicants. This research has provided useful data for researchers undertaking future studies of abalone growth, under conditions where more than one such aspect of water quality occurs, that should provide even more robust guidelines for water quality management on abalone farms.

Colt and Armstrong (1981) state that the design of a culture system for aquatic animals should be based on a knowledge of the effects of each environmental variable. The design of culture systems for Australian abalone has preceded this information, yet the culture systems satisfy the above criteria remarkably well. The development of long, narrow, lightweight raceways for culture of Australian abalone (Hone and Fleming 1997, Loipersberger 1997) satisfy the requirements of greenlip abalone for high water quality (this study) and flow rates (Fleming et al. 1997, Higham et al. 1998). Abalone in these tanks are grown in rapidly flowing, shallow water which has little potential for 'dead spots' in the water, and where partially consumed food and faeces are quickly removed. In this type of system it is unlikely that water quality would be impacted on by nitrogenous wastes or DO levels, although connecting tanks in series may cause this to occur. It is mostly where water recirculation occurs that overall levels of nitrogenous wastes or DO will reach adverse levels, and require management decisions to alleviate these potential problems.

The toxicity of each of the aspects of water quality considered in this study to greenlip abalone has often been greater than for other aquatic species. Colt and Armstrong (1981) state that levels of 0.05-0.2 mg $FAN.L^{-1}$ will cause growth reductions in most aquatic animals, while Wickins (1981) states that the maximum tolerable concentration of un-ionised ammonia is about 0.1 mg $FAN.L^{-1}$. For greenlip abalone, the EC_5 value (weight) was 0.041 mg $FAN.L^{-1}$ and a significant growth reduction occurred at 0.054 mg $FAN.L^{-1}$ (length). In comparison, the toxicity of nitrite varies greatly, and is

Table 10.1 Summarised responses of abalone to water quality variations in this study.

Toxicant	Growth of controls (SGR)	Growth reductions (p<0.05)	Significant mortalities (p<0.05)	Food consumption decrease (p<0.05)	Change in oxygen consumption (p<0.05)
Ammonia (as mg $FAN.L^{-1}$)	0.33±0.03 (W) 0.11±0.01 (L)	0.110 (W) 0.054 (L)	0.188	0.110	0.073
Nitrite (as mg NO_2-$N.L^{-1}$)	0.48±0.04 (W) 0.12±0.01 (L)	1.12, 7.80 (W) 1.12 (L)	-	-	4.29
Oxygen (as mg $DO.L^{-1}$)	0.21±0.03 (W) 0.01±0.003 (L)	5.6 (W) 6.2, 4.9 (L)	4.9	5.6	4.9
pH (greenlips)	0.87±0.11 (W) 0.29±0.03 (L)	9.01, 7.46 (W) 9.01, 7.46 (L)	6.79	-	9.25, 6.72

dependent on the chemical composition of the water (Russo 1985). In addition, the physiological differences between haemocyanin-containing aquatic invertebrates and haemoglobin-containing fish preclude any realistic comparison of nitrite toxicity, although it is generally recommended that levels for aquaculture be kept below 1.0 mg NO_2-N.L^{-1} (Wickins 1981). Greenlip abalone showed significant growth reductions at 1.12 mg NO_2-N.L^{-1} that were not further depressed until survival levels were reduced. Recommended levels of dissolved oxygen for salmonids and warmwater crustacea are to be not less that 5 mg DO.L^{-1} for more than a few hours (Wickins 1981). This study showed that similarly, DO levels below 5 mg DO.L^{-1} over extended periods produced reduced growth and survival. pH is also known to cause problems in aquaculture, especially below pH 7.7 (Wickins 1981). For greenlip abalone, the EC_5 values of 7.78 and 8.77 indicate that effects on growth are occurring above the level where many other species may be affected. Thus, greenlip abalone exhibit similar toxicity responses in many aspects of water quality with other aquatic organisms, although often demonstrating more sensitivity to these toxicants.

In considering chronic toxicity tests, previous efforts at defining safe concentrations of toxicants have been reported differently. In studies of aquatic toxicology, safe concentrations have been reported as Maximum Acceptable Toxic Concentration (MATC), which corresponds to the highest exposure concentration that does not result in significant harm to the organism in terms of survival, growth or reproduction, or the interpolation of the geometric mean of the lowest concentration having an effect and the highest concentration having no effect (Buikema et al. 1982). When aquatic toxicological studies are being related to aquaculture conditions, a more useful approach is required. The use by Wickins (1976) of EC values, or estimated concentration where growth will be reduced by a set percentage, has more application to commercial situations. This type of estimate is usually derived from a model of growth under conditions where the toxicant is at different concentrations. In this study, EC_5 and EC_{50} values have been determined for each aspect of water quality considered for chronic exposure, apart from nitrite. Thus, the more commercially useful EC_5 values can provide abalone aquaculturalists with management tools not previously

available, enabling economic control over production and water quality (Tomasso 1996).

Although growth is the most important measure for aquaculture production, other, indirect means of determining the physiological state of an organism have application in this area. Correlating biochemical or physiological changes with growth is an aim those involved in animal production are keen to achieve. However, it is apparent from this study that the aspects considered here appear at levels well above where growth is impacted, and correlate more closely with reduced survival. It is also apparent that the use of haemolymph chemistry for toxicity of growth in abalone is similar in sensitivity to histopathological changes, in that changes tend to occur at levels where survival is already compromised. Thus these two aspects can provide an indication of abalone from seriously depleted water quality, but may not be nearly as effective at indicating growth rate differences. However, these aspects are likely to retain their usefulness as means of diagnosing acutely toxic conditions, such as from acute ammonia exposure.

In comparison, oxygen consumption rate shows great promise as an indicator of metabolic stress and has been widely used to help indicate the health of animals and their overall energy expenditure or activity levels (Innes and Houlihan 1985), including abalone (Edwards 1997). Most studies of oxygen consumption rate with aquatic invertebrates have been conducted as separate, short term toxicant exposure trials. No information could be found that used a similar approach to this study, where oxygen consumption rate was measured after extended exposure, or acclimation, to toxicant(s). Despite the potential adjustments of the organisms to the toxicants over this period of acclimation, this method still demonstrated increased sensitivity over histopathology and haemolymph chemistry of these abalone in terms of detecting changes to the abalone. The use of oxygen consumption has already found wider application as a non-invasive measure of metabolic stress for abalone, as handling (Edwards 1996) and feeding (Edwards 1997) have produced metabolic changes detectable by altered oxygen consumption levels.

As a genus that uses haemocyanin as the respiratory pigment, the similarities in response to toxicants by other haemocyanin-containing organisms, most notable penaeids and freshwater crayfish, allow some conclusions to be drawn about the impact of adverse water quality on abalone. It is apparent that nitrite exposure causes severe effects for teleosts (Jensen 1996), however the impact on haemocyanin-containing organisms is much less pronounced, as abalone, for example, have an oxygen transport system directed more towards oxygen storage than oxygen delivery (Wells et al. 1998). Inorganic ions can influence haemocyanin-oxygen affinity (Mangum and Lykkeboe 1979), and toxicants such as ammonia can increase respiration rate (Smart 1978, Chen and Lai 1992). Thus the responses of haemocyanin-containing organisms may differ to those responses seen for haemoglobin-containing organisms to toxicant exposure, however, the similarities to other aquaculture species containing haemocyanin allows comparisons to be made in the absence of further data on abalone, or molluscs.

Other attempts to determine stress in abalone have had varied degrees of success. In this study, acute exposure of two populations of abalone to lethal concentrations of ammonia did not indicate differences in the response of the abalone, despite the compromised nutritional history of one of the groups. However, the use of salinity as a similar stressor has shown more promise in producing useful results that correspond with dietary history (Boarder 1997).

The various responses of body weight: shell length seen in this study suggest that this measure may actually be a sensitive indicator of physiological condition. Coote et al. (1996) showed that diet variations can produce altered shell area:body weight ratios for *H. laevigata*, while Mercer et al. (1993) detected species differences in body weight : SL ratios between *H. tuberculata* and *H. discus hannai*. Here I have shown that WWBW : SL declines from exposure to ammonia, oxygen and pH, with nitrite producing a result requiring further research. The theory put forward by Palmer (1981) that shell growth rates limit molluscan body growth rates is supported for the data here on ammonia, DO and pH, as at the more extreme toxicant levels, body weight growth rate declines more rapidly than shell length growth rates. If this theory holds, then both

shell growth rate and body growth rate are restricted by the toxicant, but in addition, shell growth rate is also restricting body growth rate, thus explaining the more rapid decline in WWBW : SL seen for ammonia, DO and pH exposure.

As a result of research in this area, further questions have been raised. The growth response pattern observed for greenlip abalone exposed to nitrite, although showing a similar response to other aquatic invertebrates that possess the respiratory pigment haemocyanin, highlights a response that warrants further attention. The rapid decrease in growth rate at very low nitrite concentrations suggests that this area could be of much greater importance to abalone production than higher concentrations, the former of which are most likely to be experienced in culture. In a similar, though not quite as dramatic, manner that ammonia affected growth at low concentrations suggests a reappraisal of this influence on abalone. As the more drastic effects of nitrite were suggested to be due to the influence of nitrite on haemocyanin, it would be greatly beneficial if the role that ions such as nitrite have on haemocyanin, especially on oxygen-loading capacity, could be considered. Future research should be undertaken in this area.

The varied degrees of success that stress tests have had for abalone indicates that more research is required in this area. The use of salinity as a short term stress has shown promise (Boarder 1997) while similar tests using ammonia showed little promise (Chapter 7). The effectiveness of oxygen consumption rate as an indicator of metabolic stress indicates that this method has a large potential for further elucidation of sub-lethal stress in abalone.

10.1 Conclusions

Greenlip abalone are sensitive to high ammonia and nitrite, low dissolved oxygen and extreme pH conditions, in terms of growth and oxygen consumption. Tissue histology provided data on alterations to gill and kidney tissue, although these occurred at levels beyond where growth was affected, at levels where survival was reduced. Similarly,

haemolymph ionic analysis proved to be relatively insensitive at detecting changes below levels at which survival was reduced.

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Appendix 1: Lengths and Weights

1. Ammonia

	Trt. level	NH3-N	Initial length	Initial weight	days	Final length	Final weight
Tank1	1	5.97135	30.99	4.32	82	34.44	5.88
Tank2	6	220.5781	30.7	4.6	81	30.55	3.83
Tank3	6	202.0225	32.76	4.77	65	33.55	4.91
Tank4	4	63.58748	32.01	4.56	85	34.36	5.77
Tank5	2	33.05628	31.14	4.16	83	33.88	5.35
Tank6	1	6.76095	31.83	4.49	82	34.77	5.83
Tank7	3	31.4195	32	4.56	82	34.4	5.8
Tank8	3	32.0446	32.44	4.76	82	34.95	6.25
Tank 9	4	51.79283	31.86	4.56	80	33.67	5.66
Tank10	1	5.930225	31.72	4.37	80	34.12	5.37
Tank11	3	31.44418	31.28	4.25	80	34.37	5.85
Tank12	6	167.7242	31.69	4.45	79	31.05	3.85
Tank13	2	22.13348	32.15	4.59	83	34.57	5.9
Tank14	2	18.94218	30.81	4.03	80	33.29	5.17
Tank15	5	110.5029	32.51	4.85	79	33.49	5.55
Tank16	5	110.3302	31.64	5.01	82	33.08	5.26
Tank17	5	124.5265	31.68	4.44	84	33.26	5.3
Tank18	4	55.06638	31.94	4.55	85	33.96	5.64

2. Nitrite

	Treatment	NO_2-N mg/L	Initial length	Initial weight	Days	Final length	Final weight
Tank 1	1	0.0162	34.79	5.547	79	37.75	7.7
Tank 2	6	7.8171	33.59	4.923	70	34.13	6.05
Tank 3	6	8.1066	34.33	5.238	52	34.62	5.86
Tank 4	4	1.8249	34.5	5.372	67	35.77	6.38
Tank 5	2	0.5954	34.11	5.327	58	35.64	6.34
Tank 6	1	0.013	35.13	5.717	73	38.93	8.48
Tank 7	3	1.133	35.8	6.005	51	37	7.34
Tank 8	3	1.1043	34.55	5.452	66	36.43	6.75
Tank 9	4	1.8281	30.08	5.593	73	37.88	7.74
Tank 10	1	0.0147	35.46	5.743	74	38.76	8.14
Tank 11	3	1.135	34.14	6.359	60	36.01	6.68
Tank 12	6	7.3156	34.91	5.506	70	35.4	6.59
Tank 13	2	0.5362	34.57	5.402	66	36.74	6.85
Tank 14	2	0.552	34.47	5.362	66	36.65	6.63
Tank 15	5	4.3895	35.13	5.689	70	37.68	7.9
Tank 16	5	4.0575	36.43	6.255	70	37.62	7.9
Tank 17	5	4.0549	35.94	6.145	60	37.24	7.58
Tank 18	4	2.0321	35.81	6.079	55	36.97	7.44

3. Dissolved oxygen

Tank	Treatment	Mean DO (mg/L)	Initial length	Initial weight	Days	Final length	Final weight
1	5	4.772816	44.68919	10.86865	60	44.85	11.42
2	1	8.834646	44.82258	11.30933	68	45.39	12.91
3	2	7.654839	44.14595	10.64811	70	44.43	12
4	1	8.910236	43.04595	9.85	72	44.06	11.67
5	4	5.416514	43.8027	10.60135	64	43.69	11.52
6	4	5.688991	45.51667	11.55967	64	45.29	12.88
7	5	4.841748	43.79189	10.53946	61	43.36	10.42
8	5	4.962745	43.42414	10.32241	50	43.75	10.72
9	2	7.6792	44.43793	11.11379	69	44.28	13.13
10	6	4.213402	44.05405	10.79459	59	43.08	10.09
11	4	5.544037	43.69375	10.56719	64	43.68	11.58
12	1	9.014173	44.85882	11.01882	75	45.42	12.53
13	3	5.996667	44.28125	10.86844	68	44.58	11.74
14	3	6.078333	43.90313	10.70969	69	44.24	11.15
15	2	7.668	44.08387	10.95226	72	44.57	12.87
16	6	4.242268	44.08286	11.03943	57	43.77	10.72
17	3	6.454167	43.82813	10.60281	71	44.04	11.41
18	6	4.274227	43.54571	10.41286	57	43.88	10.31

4. Acute ammonia exposure

Tank	Diet	Ammonia mg FAN/L	Initial length	Initial weight	% mortality @ 130 hrs
1	Heated	0.003333	56.125	22.05667	8.333333
2	Normal	0.361	54.71667	20.815	0
3	Heated	0.361	54.18333	20.59167	0
4	Normal	0.361	58.45833	24.84417	0
5	Heated	0.003333	55.41667	21.27417	0
6	Normal	0.998	56.18333	21.69667	0
7	Normal	0.998	57.65833	23.50417	0
8	Heated	0.361	57.63333	21.8	0
9	Heated	0.998	58.10833	24.89917	0
10	Normal	0.656333	55.15	21.26333	33.33333
11	Heated	0.656333	53.48333	20.39417	100
12	Normal	0.003333	56.74167	23.06667	0
13	Normal	0.656333	54.94167	21.32667	91.66667
14	Normal	0.003333	56.09167	22.28333	16.66667
15	Heated	0.656333	52.35833	19.21667	41.66667
16	Heated	0.998	53.45833	20.1425	0

5. pH

Treatment	Tank	Initial length	Initial weight	Days	Final length	Final weight
1	4	27.88	2.57	57	29.98	3.39
1	12	27.01	2.42	57	30.03	3.57
1	13	26.78	2.34	46	30.58	3.64
2	3	26.78	2.34	57	31.21	3.63
2	16	26.89	2.45	60	31.38	3.83
2	17	27.16	2.43	60	33.38	4.72
3	8	23.97	1.75	48	26.98	2.68
3	14	24.38	1.85	59	27.81	2.67
3	15	24.13	1.80	58	27.63	2.73
4	2	25.81	2.15	55	27.59	2.71
4	9	25.77	2.12	64	28.29	2.82
4	10	25.46	2.01	66	26.89	2.41
5	5	26.69	2.39	63	27.66	2.58
5	7	27.93	2.62	64	29.57	3.05
5	18	27.08	2.38	62	27.88	2.73
6	1	28.31	2.83	48	28.3	1.92
6	6	27.26	2.43	41	27.3	
6	11	27.02	2.36	43	27.07	

Appendix 2: Specific growth rates of abalone in chronic ammonia trial, treatment 6.

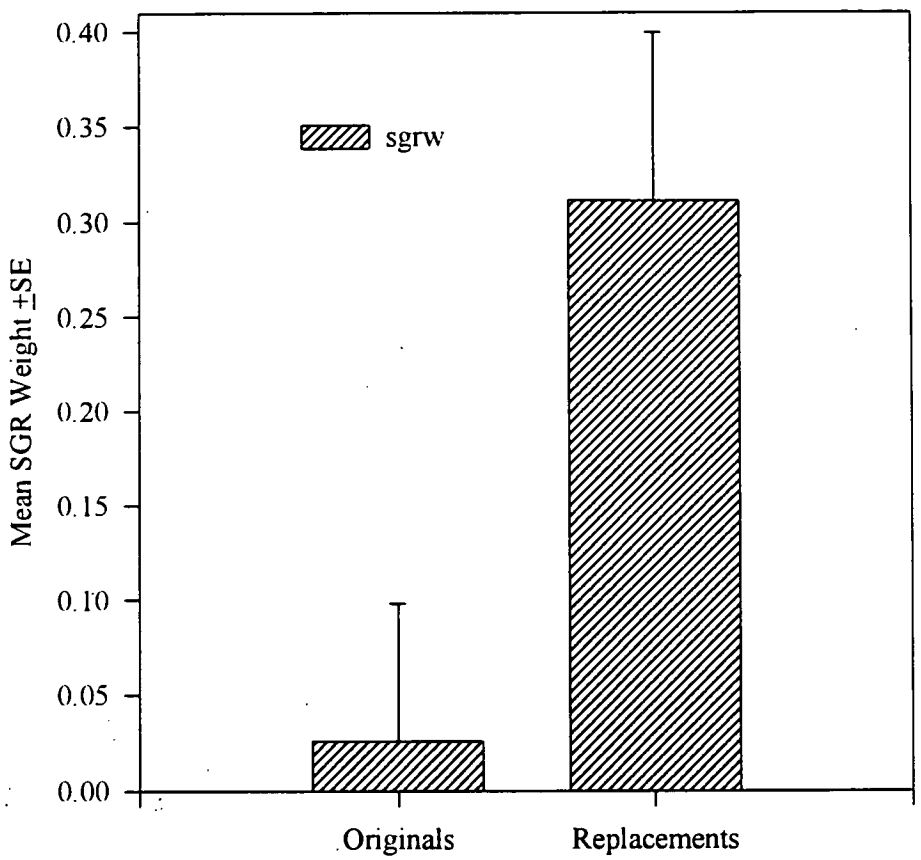

Figure A2.1 Mean Specific growth rates (weight) of abalone in treatment 6, chronic ammonia exposure trial.

In chapter 3, abalone chronically exposed to ammonia at a mean concentration of 0.188 mg FAN.l^{-1} experienced significant mortality within the first third of the trial. During this time, average daily ammonia concentration was 0.363 mg FAN.l^{-1}, which was subsequently reduced for the duration of the trial to 0.159 mg FAN.l^{-1}, to give a total average of 0.188 mg FAN.l^{-1}. To avoid compounding errors due to differences in

stocking density, more abalone from the initial population were added to the cages of treatment 6. The growth rates for the original abalone, at the higher concentration, and the replacement abalone, grown at a reduced concentration are given in Figures A2.1 and A2.2. No significant differences were noted for either weight or length, in terms of SGR.

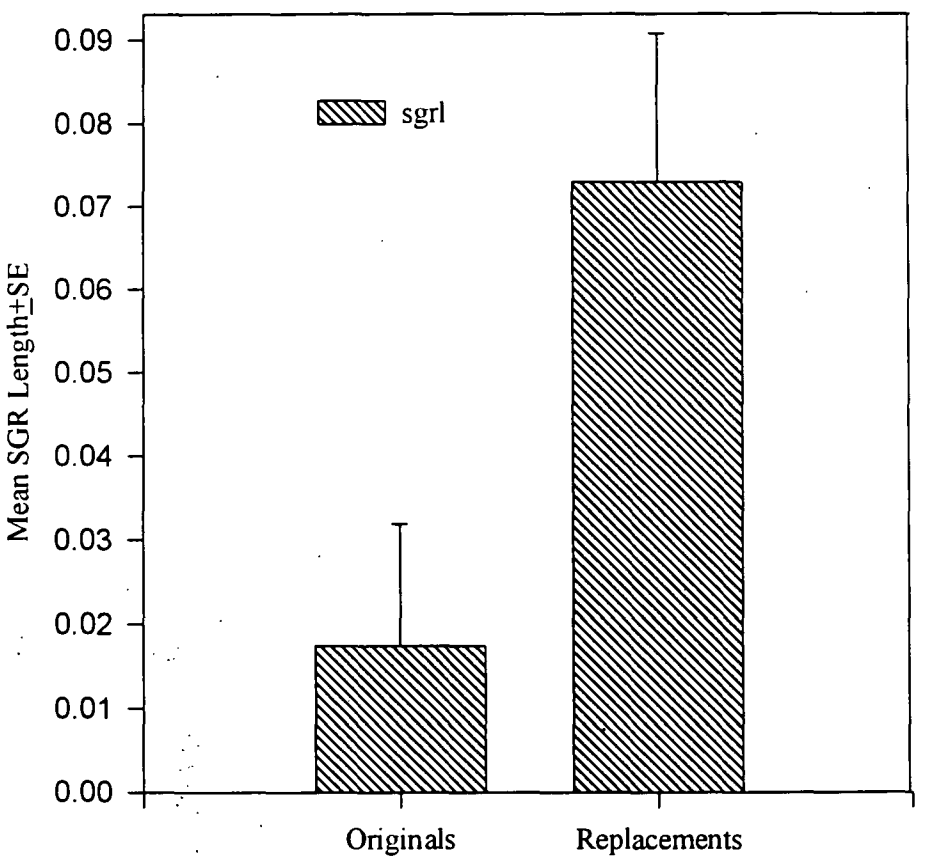

Figure A2.2 Mean Specific growth rates (length) of abalone in treatment 6, chronic ammonia exposure trial.

Appendix 3: Diets

1. Food Leaching Rate Experiment

Introduction:

The food consumption rates for the bioassays included in this thesis were estimated as apparent food consumption rate (dry weight food added less dry weight of food remaining). No corrections were made for soluble or particulate losses at this stage. This experiment serves to supplement this information with an investigation into the losses occurring in seawater over time. In addition, the effect of pH on food leaching rate will also be assessed.

Aims:

To assess the amount of abalone food leaching into seawater, over a 3 day period, at 3 pH levels.

Materials and Methods:

This experiment was conducted in three 60 L containers. The diet used for some of the bioassay experiments was FRDC Diet #2, as produced at SARDI, and the remainder used FRDC Diet #6, as produced at MSH. A sample of diet from SARDI was obtained (FRDC Diet #6), however, this diet appeared markedly different to previous samples. A sample of the FRDC Diet #6 as made at MSH was also used for comparison. The pH levels were 8.89, 8.35 and 7.14, with triplicates of each diet at each level. An initial sample was set aside and 54 samples of food (10.1g each) were placed in the tanks, and were removed from the containers each day for three days. Food samples were dried at 55°C overnight and weighed.

Data were subjected to one factor ANOVA after meeting assumptions of normality using the Shapiro-Wilk test (Zar, 1996) and homogeneity of variance using Cochran's test (Underwood, 1981). Replicates were considered to be independent and pH level, days and diet were analysed as fixed factors. All analyses were conducted using JMP 3.0 software (SAS Institute).

Results:

The was no significant relationship between dry weight remaining and day of removal or with dry weight remaining and pH. However, a significant difference in dry weight remaining occurred between the two diets ($p<0.001$). The SARDI diet had an average recovery of 91.4% over the three days, while the MSH diet had an average recovery of 98.6%.

Discussion:

The two diets showed different leaching rates during the experiment. The diet produced at MSH was of the flat, pasta-type pellets, most commonly used for abalone food. The SARDI diet sample was quite yellow, and consisted of much smaller fragments than the MSH food. The MSH diet sample was much more representative of the food supplied to the abalone during bioassay experiments. The minimal loss rate that occurred with the MSH diet sample suggests that the error within the calculations of food consumption rate due to soluble and particulate losses are minimal, in the order of 1.5%.

2. Diet analysis

Method as used by Allison Laboratories, Hobart for determining water soluble protein in fish meal:

10.0 g of fish meal is scaled off exactly and added 200 ml water in a 250 ml glass flask/bowl. This bowl is heated in boiling water for 30 minutes, stirred occasionally. The mixture is cooled and made up to 250 ml. The mixture is filtered in a medium mesh filter (no. 588). From the filtrate a 50 ml sample is instantly added H_2SO_4 and Hg and must be distilled according to the Kjaldahl method. Potassium sulphate is added after the water has been evaporated to prevent foam. The water soluble protein is calculated in percentage from the total protein content.

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EFFECTS OF CHRONIC EXPOSURE OF GREENLIP ABALONE, *HALIOTIS LAEVIGATA* DONOVAN, TO HIGH AMMONIA, NITRITE, AND LOW DISSOLVED OXYGEN CONCENTRATIONS ON GILL AND KIDNEY STRUCTURE

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ABSTRACT After chronic, sublethal bioassays of juvenile greenlip abalone, *Haliotis laevigata* Donovan, to reagent ammonia, nitrite, and low dissolved oxygen, tissue samples were dissected for histological analysis. Exposure to the highest ammonia treatment (0.188 mg of free ammonia-nitrogen ([FAN]L^{-1})) resulted in little difference to the gills of these abalone, relative to the controls (0.006 mg of FAN L^{-1}), whereas at this concentration, the right kidney showed decreased tubule definition and enlarged tubule lumen. Exposure to 7.8 mg of NO_2-N L^{-1}, resulted in gill lamellar thickening and epithelial lifting along with a proliferation of mucous cells. The proportion of kidney cell contents occupied by granules increased at this nitrite concentration. Associated with this change in kidney structure was an increase in basally located eosinophilic cytoplasm. Gill mucous cells from abalone exposed to depressed dissolved oxygen levels (55% oxygen saturation) exhibited more intense staining, indicative of a change in mucous composition. Some necrosis of gill epithelium was evident, either as a result of or in association with the occurrence of ciliates (Ancistrocomidae) between the gill lamellae. Right kidney tissue did not exhibit any obvious changes in relation to exposure to low dissolved oxygen levels. Chronic exposure to slight oxygen supersaturation (117%) caused no apparent effects on gill or kidney structure.

KEY WORDS: abalone, *Haliotis laevigata*, ammonia, nitrite, oxygen, histology

INTRODUCTION

The present expansion of abalone aquaculture (Hone and Maguire 1996) brings with it the likelihood of encountering suboptimal water quality, especially where recirculating aquaculture systems are used (Jirsa et al. 1997). The culture of animals in nonoptimal environments may result in deaths, as a direct result of one or more components of the environment or from infectious diseases activated indirectly by suboptimal environments, or in decreased productivity (Tomasso 1996).

The external environment can, if suboptimal, produce deleterious changes in aquatic animals. The overt signs of toxicity are nearly always preceded by biochemical, physiological, and/or morphological changes in the organism (Meyers and Hendricks 1985). Often, the gills are among the organs most affected by waterborne pollutants (Mallat 1985), because the respiratory surface provides an extensive interface with the aquatic environment. In many fish, the kidney often forms a site of histological changes in response to toxicants (Russo 1985). Abalone are diotocardians, possessing two kidneys of differing functions. The role of the right kidney is believed to be in nitrogen excretion because of the presence of excretory vacuoles (Andrews 1985). Some resorption of solutes, which is also the main function of the left kidney, is also believed to occur, because of the presence of coated pits opening between microvilli (Voltzow 1994). The role of the right kidney in nitrogen excretion and protein turnover suggests a possible role with the toxicants considered in this study.

The purpose of this study was to bring together histological observations of juvenile greenlip abalone, *Haliotis laevigata*, from three chronic, sublethal bioassay studies. Specifically, the effects of sublethal exposure for 2–3 mo to high ammonia, nitrite, or low dissolved oxygen on gill and right kidney histological structure were investigated.

MATERIALS AND METHODS

Juvenile greenlip abalone were sampled after chronic sublethal exposure for 58–82 days to ammonia (as NH_4Cl) (Harris et al. 1998), nitrite (as $NaNO_2$) (Harris et al. 1997), or low dissolved oxygen (Harris et al. in press). The experimental ranges were 0.006–0.188 mg, free ammonia-nitrogen (FAN), L^{-1} (0.237–9.04 mg total ammonia-nitrogen L^{-1}), 0.024–7.80 mg NO_2-N L^{-1} and 8.9–4.2 mg of dissolved oxygen L^{-1} (117–57% dissolved oxygen saturation).

Five abalone were sampled from two of the triplicate bioassay tanks for each treatment and bled, via an incision in the foot, for 2–3 min before being dissected to remove the posterior portion of the viscera containing the gills and kidney. This tissue was fixed in phosphate-buffered formalin at room temperature (15–18°C) and then dehydrated through a graded ethanol series to xylene in a Tissue-Tek II tissue processor. Dehydrated tissue samples were embedded in paraffin resin on a Shandon Histocentre 2 and sectioned on a Microm HM 340 microtome at 4 μm. Routine Harris' hemotoxylin and eosin staining was carried out on all tissues processed with a Shandon Linistain GLX automatic tissue stainer. All sections were mounted in DPX and examined under a light microscope.

RESULTS

Exposure to the highest ammonia treatment (0.188 mg of FAN L^{-1}) resulted in little difference to the gills of these abalone, rela-

tive to the controls (0.006 mg of FAN L^{-1}) (Fig. 1). At this treatment level, however, the right kidney of all sampled abalone showed less definition in the tubules, and the lumen of the tubules appeared enlarged (Fig. 2). At 0.110 mg of FAN L^{-1}, 10% of the sampled abalone showed both reduced right kidney definition and an enlarged lumen, whereas 20% of abalone showed reduced right kidney definition and 20% of abalone exhibited enlarged right kidney lumen. Typical kidney structure for abalone not exposed to elevated levels of ammonia or nitrite or to low dissolved oxygen is shown in Figure 3.

From exposure to 7.8 mg of NO_2-N L^{-1}, thickening of the lamellae and epithelial lifting of gills were evident in all observed abalone, along with a proliferation of mucous cells at the junction of the lamellae and central gill axis (Fig. 4). Mucous cells, common at the distal tip of the lamellae, also extended further toward the base of the lamellae than for other treatments and control abalone. These mucous cells are evident both as complete and apparently discharged cells with adhered mucous, often giving a ragged appearance to the lamellae and contributing to the poor brush border definition observed here (Fig. 5). Abalone exposed to concentrations less than 7.8 mg of NO_2-N L^{-1} showed typical gill structure including the principal gill filament and lamellar junction (Fig. 6) and lamellar tip (Fig. 7). At 4.15 mg of NO_2-N L^{-1}, 20% of sampled abalone showed thickened gill lamellae, 10% showed lifting of gill epithelium, and 40% showed a proliferation of gill mucous cells. At the highest nitrite concentration (7.8 mg of NO_2-N L^{-1}), the overall height of the kidney tubule cells was increased. This was due to an increase in both the amount of pigment granules in the supranuclear region (toward the lumen surface) and the amount of eosinophilic (protein rich) cytoplasm located in the subnuclear region toward the base membrane (Fig. 8).

Gill mucous cells from abalone exposed to depressed dissolved oxygen levels (55% oxygen saturation) exhibited more intense staining, indicative of a change in the composition of mucous. Necrosis of gill epithelium was evident in all sampled abalone at this dissolved oxygen concentration, either as a result of or in association with the occurrence of ciliates (family Ancistrocomidae; D. Lynn pers. comm.) between the gill lamellae (Fig. 9), observed in 80% of sampled abalone at this concentration. At 63% of oxygen saturation, necrosis of gill tissue was evident in 20% of sampled abalone, whereas ciliates were observed in 40% of the abalone. Right kidney tissue did not exhibit any obvious changes in relation to exposure to low dissolved oxygen levels. One treatment was maintained at 117% oxygen saturation, and no adverse effects on gill or kidney structure were evident.

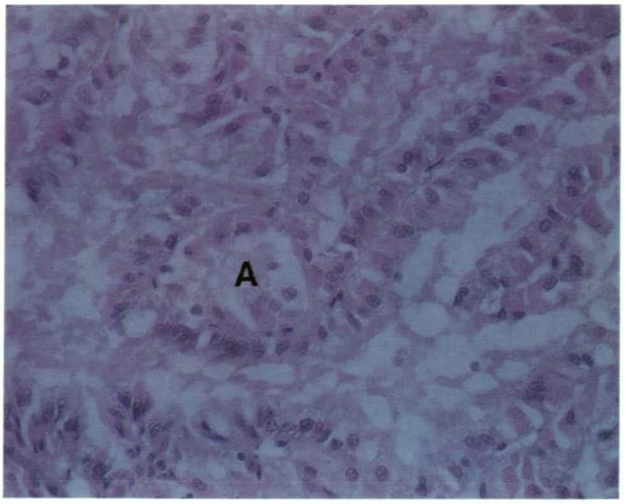

Figure 2. Right kidney of abalone exposed to 0.188 mg of FAN L^{-1}. Magnification, ×400. (A) Enlarged lumen of kidney tubule.

DISCUSSION

Some effects of ammonia on the histological structure of fish have been documented (see Russo 1985), although data for invertebrates are less common. FAN levels of 0.04–0.4 mg L^{-1} have been shown to induce inflammation and degeneration of gills and kidneys for a variety of fish species (Russo and Thurston 1991). The swollen, rounded secondary lamellae observed in rainbow trout after long-term exposure to ammonia (Smart 1976) were not observed in this study. However, among fish, the effects of ammonia are varied, because not all authors found hyperplasia and/or other degenerative changes to the gill structure. Sublethal ammo-

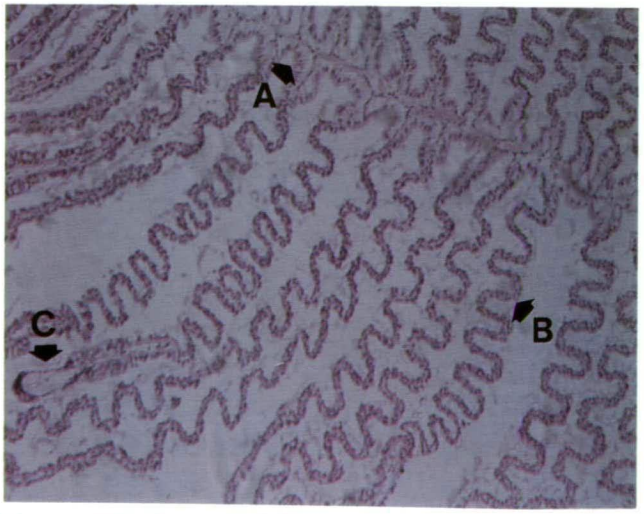

Figure 1. Gills of control abalone from ammonia bioassay. Magnification, ×100. (A) principal filament; (B) gill lamella; (C) distal tip of gill lamella.

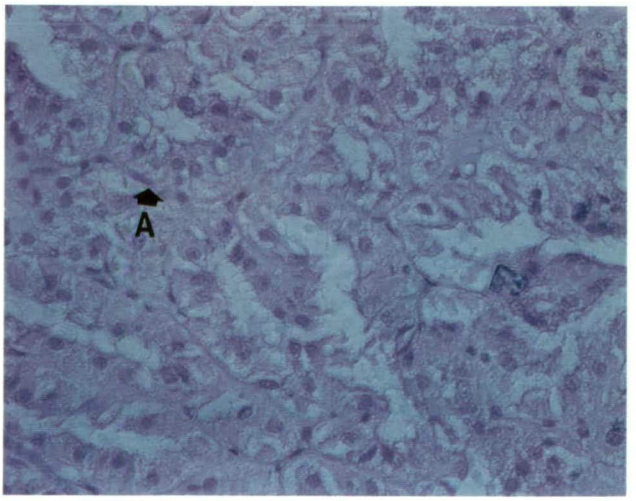

Figure 3. Right kidney of control abalone from nitrite bioassay. Magnification, ×400. (A) Exterior perimeter of normal kidney tubule.

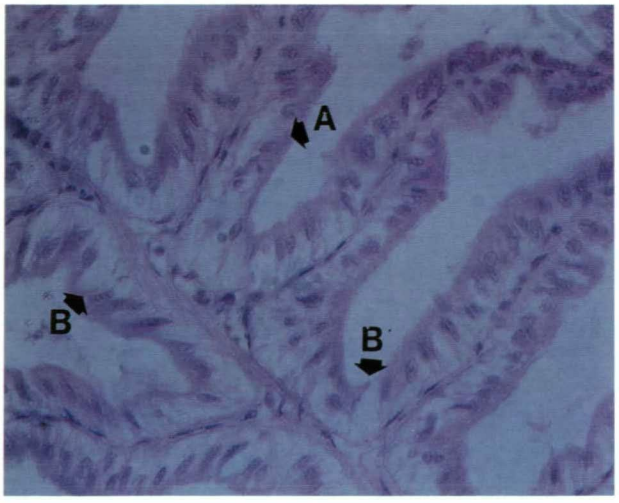

Figure 4. Distal tip of gill lamellae exposed to 7.80 mg of NO_2-N L^{-1}. Magnification, ×400. (A) Lifting of epithelium; (B) proliferation of mucous cells.

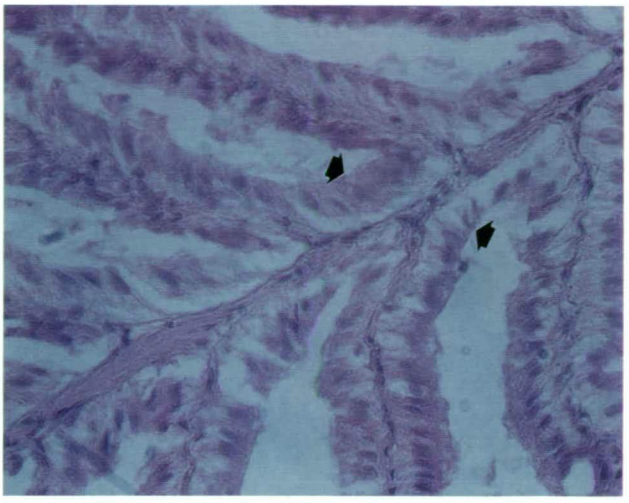

Figure 6. Junction of gill lamellae and principal filament of control abalone from nitrite bioassay. Magnification, ×400.

nia levels are also known to cause histological changes in the kidneys of many fish (Colt and Armstrong 1981). The observed differences in cell definition indicate that external ammonia had some effect on kidney structure in greenlip abalone, even though survival at this concentration was under 50% for a minimum of 58 days of exposure (Harris et al. 1998).

The long term effects of nitrite on histological structure have not been well documented for aquatic invertebrates. Nitrite is known to bioaccumulate in gill, liver, brain, and muscle tissue of fish (Margiocco et al. 1983) and to increase susceptibility to diseases (Hanson and Grizzle 1985). Michael et al. (1987) reported gill hypertrophy and hyperplasia in *Clarias lazera,* with some degree of gill epithelial lifting and necrosis. Gill degeneration, observed in rainbow trout within 3 wks of exposure to nitrite, was noted to disappear with increasing exposure time (Wedemeyer and Yasutake 1978). The observed changes in number and location of mucous cells in greenlip abalone gills suggest a hypersecretion of mucous as a response to chronic sublethal nitrite exposure at 7.8 mg of NO_2-N L^{-1} for a minimum of 82 days of exposure. Although these changes were more evident than for exposure to ammonia, a survival rate of 73% indicates that these changes may not affect survival as much as growth rate (Harris et al. 1997). The observed increase in right kidney pigment and granule deposition may be a reflection of increased kidney protein, and hence cell, turnover. Arillo et al. (1984) hypothesized that tissue hypoxia, as a result of nitrite exposure, was the contributing factor to acute toxicity for rainbow trout. The tissue hypoxia of fish is mediated through production of methemoglobin from the respiratory pigment hemoglobin. Because this pigment does not occur in abalone, not surprisingly, the lesions seen with nitrite toxicity differ from those seen with anoxia.

The effects of low dissolved oxygen on abalone in this study have some parallel in the literature. Histopathological effects of oxygen supersaturation to the red abalone, *Haliotis rufescens* (Elston 1983, Elston and Lockwood 1983) included the presence of

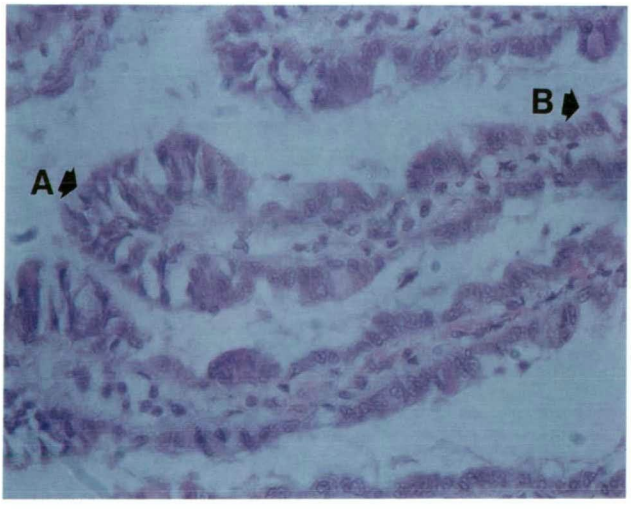

Figure 5. Junction of gill lamellae and principal filament of abalone exposed to 7.80 mg of NO_2-N L^{-1}. Magnification, ×400. (A) Complete mucous cell; (B) discharged mucous cell with adhered mucous.

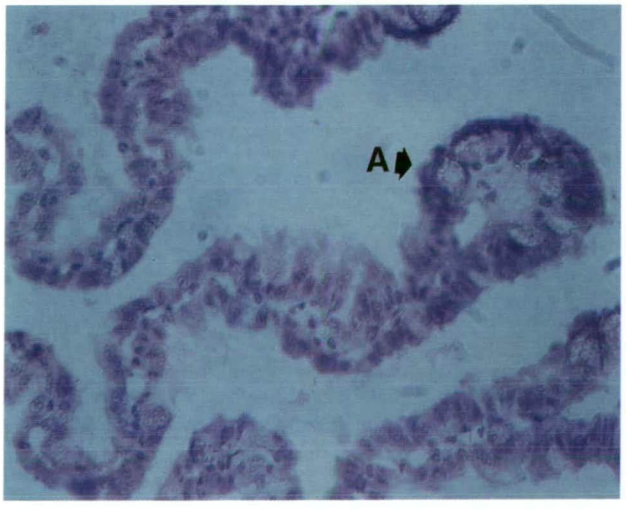

Figure 7. Distal tip of gill lamellae from control abalone from nitrite bioassay. Magnification, ×400. (A) Normal gill lamellar tip showing mucous cells.

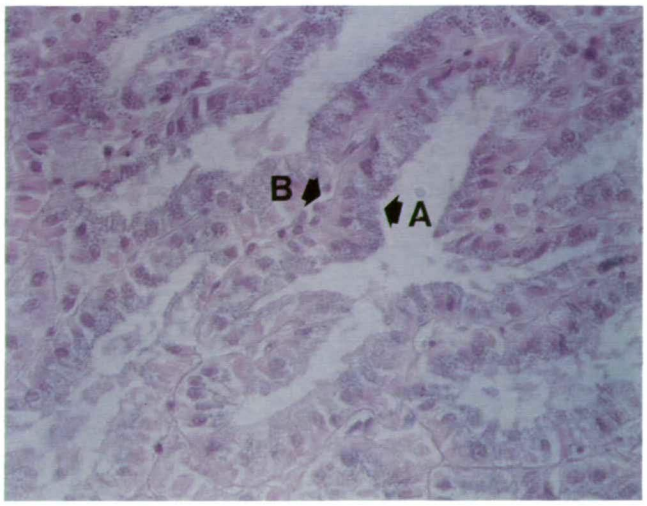

Figure 8. Right kidney of abalone exposed to 7.80 mg of NO_2-N L^{-1}. Magnification, ×400. (A) Pigment granules in supranuclear region of kidney tubule cell; (B) eosinophilic cytoplasm in subnuclear region of kidney tubule cell.

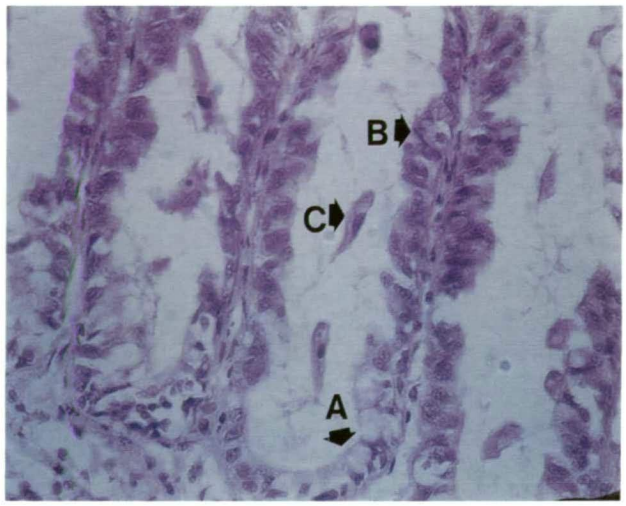

Figure 9. Junction of gill lamellae and principal filament of abalone exposed to 55% oxygen saturation. Magnification, ×400. (A) Intensely stained gill mucous cells; (B) necrotic tissue; (C) ciliate between gill lamellae (family Ancistrocomidae).

gaseous emboli in epipodial, oral, and pedal tissues. Elston (1983) reported the formation of these emboli at 150% oxygen saturation, whereas at 117% oxygen saturation, no emboli were evident in the abalone sampled in this study. Leitman (1992) reported increased bacterial counts from effluent water of abalone tanks at 143% oxygen saturation. The occurrence of ciliates at the 55% oxygen saturation treatment suggests an increased susceptibility to disease at depressed oxygen saturation. Walters and Plumb (1980) determined that low dissolved oxygen levels increase the susceptibility of channel catfish to bacterial infection. Survival of abalone also decreased to 59% of controls at this concentration (Harris et al. in press).

Some of the responses seen to the toxicants in this study are representative of the situation for many other aquatic animals. Mallat (1985) reviewed the literature on structural changes, in fish gills, induced by toxicants and determined that histopathological gill lesions are largely nonspecific in nature, with changes in gill epithelium, bulbing or fusing of gill lamellae, hypersecretion and proliferation of mucocytes, and changes in chloride cells and gill vasculature common to many different exposure conditions. Mallat (1985) also determined that the frequency of gill lesions is greater from acute rather than sublethal exposure and in freshwater situations rather than marine. This may explain the lack of effect of ammonia on the abalone gills and the subtle nature of changes has occurred during sublethal exposure of greenlip abalone to ammonia, nitrite, and low dissolved oxygen.

In this study, we have identified different histological changes for each environmental stressor, but the effects with low dissolved oxygen may have been dependent on ciliate infestation. Future research will involve bioassays for combinations of stressors and also a broadening of stress attributes to include biochemical changes. The overall aim is to establish a set of stressor-specific changes that can be used for diagnostic purposes.

ACKNOWLEDGMENTS

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EFFECT OF NITRITE ON GROWTH AND OXYGEN CONSUMPTION FOR JUVENILE GREENLIP ABALONE, *HALIOTIS LAEVIGATA* DONOVAN

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ABSTRACT Juvenile greenlip abalone, *Haliotis laevigata* Donovan (mean whole weight, 5.61 g) were grown for 2–3 mo in bioassay tanks. Specific growth rate (SGR), measured on a whole-weight ($p < 0.05$) or shell length ($p < 0.01$) basis, was significantly affected by nitrite ($NaNO_2$). Modeling of the whole weight indicated relatively uniform growth depression (average SGR weight of 67.2% relative to the control, 0.024 mg of NO_2-N L^{-1}), regardless of concentration in the range of 0.56–7.80 mg of NO_2-N L^{-1}. This pattern of growth depression, which is independent of nitrite concentration once growth is reduced relative to controls, has been recorded by other researchers for penaeid shrimp and freshwater crayfish. SGR data for shell length exhibited a similar pattern, except that much more severe growth depression (average SGR length of 17.7% relative to the control) was recorded for the highest concentration (7.80 mg NO_2-N L^{-1}). Compared with several aquatic species studied by other authors, greenlip abalone are sensitive to nitrite on a growth basis. Oxygen consumption declined sharply with increasing nitrite concentration ($y = 82.452.e^{-0.154.x}$; range, 0.025–7.72 mg of NO_2-N L^{-1}). However, neither food consumption (as a percentage of initial biomass, corrected for mortality) nor survival was significantly affected by nitrite concentration ($p > 0.05$).

KEY WORDS: abalone, *Haliotis laevigata*, nitrite, growth molluscs, oxygen

INTRODUCTION

The major source of nitrogenous compounds in aquaculture systems is usually from the catabolism of protein contained within feed, with ammonia being the major end product (Colt and Armstrong 1981). In aerobic environments, nitrifying bacteria oxidize ammonia to nitrogen oxides, including nitrite (by *Nitrosomonas* spp.), and finally nitrate (by *Nitrobacter* spp.) (Brock and Madigan 1991). In flow-through systems, ammonia will be the principal toxic metabolite by-product, but in recirculating systems, both ammonia and nitrite may occur at toxic levels (Colt and Armstrong 1981). As the end product of nitrification, nitrate is the least toxic of inorganic nitrogen compounds to juvenile aquatic animals and will only be a problem in recirculating systems because of its effects on osmoregulation (Colt and Armstrong 1981). The conversion of nitrite to nitrate can be the rate-limiting step when conditioning biofilters, as a build-up of ammonia can inhibit this conversion and cause a subsequent build-up of nitrite (Anthionsen et al. 1976, de Guingand and Maguire 1992).

As a component of nitrogenous wastes, nitrite is known to affect oxygen transport (Jensen et al. 1987, Jensen 1995), cause tissue damage (Michael et al. 1987), and result in the oxidation of other compounds (Crawford and Allen 1977, Colt and Armstrong 1981). Nitrite penetrates cell membranes and is bioaccumulated in the extracellular spaces, particularly in gill, liver, brain, and muscle tissues of fish (Jensen 1995). The ionized form of nitrite, although not freely diffusible, is actively transported across gill membranes (Wedemeyer and Yasutake 1978, Bath and Eddy 1980, Jensen 1995, Schoore et al. 1995).

In fish, nitrite combines irreversibly with hemoglobin to cause methemoglobinemia because the hemoglobin is no longer able to combine with oxygen (Needham 1961). Hemocyanin is the respiratory pigment in some invertebrates including abalone, and although there is some suggestion of nitrite forming a complex with hemocyanin thereby affecting oxygen consumption, others consider this effect to be negligible (Needham 1961), or less deleterious than the complexing of nitrite and hemoglobin (Jensen 1995). Hemocyanin can take up oxygen even in the presence of strong oxidizing agents (Needham 1961), and hence, oxygen transport by hemocyanin is generally much less affected by nitrite than is oxygen transport by hemoglobin. The formation of methemocyanin occurs primarily at low pH, in the presence of a large excess of nitrite, and appears unimportant at physiological pH (Jensen 1995). However, a severe excess of nitrite may occur in some aquatic animals, because nitrite accumulates in extracellular fluid in freshwater fish and crustaceans in concentrations well above ambient (3–33 times higher in 1–7 days) (Gutzmer and Tomasso 1985, Jensen et al. 1987, Harris and Coley 1991, Schoore et al. 1995, Jensen 1996, Stormer et al. 1996), although this has not yet been established for abalone. The competitive exclusion of nitrite ion uptake via the chloride cells by chloride ions increases the tolerance of marine fish to nitrite (Wedemeyer and Yasutake 1978) while also preventing extracellular nitrite levels of marine fish and crustaceans from greatly exceeding ambient concentrations (Eddy et al. 1983, Chen and Chen 1992a, Chen and Chen 1992b, Jensen 1996).

Although data on effects of nitrite on crustaceans and fish are readily available, information regarding molluscs, including abalone, is limited. Abalone culture is increasing in response to declining worldwide fishery production (Hone and Maguire 1996). Increasing production and subsequent reliance on protein-rich, formulated feeds and the introduction of recirculating culture systems increase the likelihood of abalone encountering elevated nitrite concentrations. In this study, we aimed to assess the chronic toxicity of nitrite to juvenile greenlip abalone, *Haliotis laevigata*, the most widely farmed abalone in Australia, in terms of growth and oxygen consumption.

TABLE 1.

Abbreviations used in the text.

Abbreviation	Definition
NO_2-N	Nitrite nitrogen
SGRW	SGR (WWBW% day^{-1}) $\frac{\ln(\text{final weight}) - \ln(\text{initial weight})}{\text{days}} \times 100$
SGRL	SGR (shell length % day^{-1}) $\frac{\ln(\text{final length}) - \ln(\text{initial length})}{\text{days}} \times 100$
SE	Standard error

All values given as mean ± SE, unless otherwise stated.

MATERIALS AND METHODS

The juvenile greenlip abalone used in these experiments were approximately 3 y old, from a commercial hatchery at Bicheno, Tasmania, Australia, where the research was conducted (148′18″E, 41′53″S). The initial mean length and weight of the abalone were 35.0 ± 0.1 mm (mean ± SE) and 5.61 ± 0.06 g (mean ± SE) (n = 719). For 2–3 mo before experimentation, these abalone were maintained on a mixture of three formulated abalone feeds (ABCHOW, Deakin, Promak) and benthic diatoms. Abalone used for this experiment were relaxed with aerated warm water (23–25°C) until they could be easily removed from tank surfaces. Subsequently, they were tagged (Hallprint, Adelaide, Australia) and then randomly distributed to 18 bioassay units after being blotted and weighed to the nearest 0.01 g (whole wet body weight, WWBW) and measured with callipers to 0.1 mm for determining growth indices (Table 1). Most of the abalone were exposed to specific nitrite (NO_2^-) concentrations for 82 days and then again weighed and measured to assess individual growth.

Bioassay System

Abalone were held in cages (100 mm diameter × 35 cm polyvinyl chloride tubes, with 6-mm mesh floor and 8-mm mesh wall sections) suspended vertically within 70-L bioassay tanks (Harris et al. in press). Forty abalone were contained within a single cage in each tank. Oceanic seawater was filtered through a commercial sand filter and delivered to six 1,100-L reservoirs. The reservoirs were drained and refilled each day with seawater dosed with the appropriate amounts of sodium nitrite ($NaNO_2$). Each reservoir was connected to a constant head chamber, which supplied three bioassay chambers via standard lengths of black 4-mm polypropylene tubing, which were replaced fortnightly to avoid nitrification by biofilms (Harris et al. in press). The bioassay tanks had conical ends to concentrate solid wastes and to avoid air spaces. Daily flow rates averaged 181.8 ± 1.5 mL min^{-1} (mean ± SE; n = 54), giving an effective replacement rate of 90% of bioassay tank volume in 12 h, in accordance with Sprague's (1969) 90% recommended replacement in 8–12 h. The experiment was conducted with 200- to 300-W aquarium heaters in the bioassay tanks and head adjustment columns, respectively, to maintain a constant temperature (Table 2). The average daily recovery of NO_2-N between reservoirs and bioassay tanks varied from 84.0 to 95.6% (n = 5).

Water Quality Analysis

The nitrite concentration of one replicate tank from each treatment, along with pH, temperature, and dissolved oxygen in all tanks were measured on each of 72 days. Water samples were collected in acid-washed glassware, and nitrite was measured by the diazotization method (Grasshoff 1989). A pH meter and combination glass electrode (Hanna Instruments HI 9025) were calibrated with phosphate (pH = 7.00) and borate (pH = 9.28) buffers daily before use (Bruno and Svoronos 1989). Ammonia was measured occasionally by the indophenol blue spectrophotometric method (Dal Pont et al. 1974). A WTW Microprocessor Oximeter 0XI 96 oxygen electrode, used for daily measurements, was calibrated before use in "air-saturated" seawater and checked periodically using Winkler's titration.

Experiment 1: Chronic Nitrite Exposure

One control and five experimental treatments were established (Table 2); average nitrite concentrations ranged from 0.024 to 7.80 mg of NO_2-N L^{-1}. All cages were checked daily for mortality.

All tanks were fed a proprietary, formulated abalone diet (ABCHOW; 35% protein on a dry matter basis) every 2–3 days. The feeding ration was adjusted in response to food consumption data as the trial progressed. Food consumption was estimated on four occasions (Days 16, 38, 60, and 63) from uneaten food removed from the base of the cages after 2 days and dried for 24–48 h at 55–60°C. Residual food weight was not corrected for soluble

TABLE 2.

Water quality parameters within the chronic nitrite exposure trial (Experiment 1).

Treatment	NO_2-N mg L^{-1}			pH	% Survival	Food Consumption (g g^{-1} day^{-1})
		Min	Max			
1	0.024 ± 0.005	0	0.45	7.94	100 ± 0	0.037 ± 0.001
2	0.56 ± 0.018	0.36	1.68	7.90	89.17 ± 10.83	0.035 ± 0.001
3	1.12 ± 0.017	0.49	1.43	7.88	66.67 ± 18.05	0.043 ± 0.006
4	1.87 ± 0.059	0.61	2.74	7.88	77.5 ± 16.65	0.032 ± 0.002
5	4.15 ± 0.094	1.89	5.63	7.92	90.83 ± 9.17	0.034 ± 0.002
6	7.80 ± 0.233	0	10.66	7.91	73.37 ± 21.74	0.052 ± 0.016

* Values are means ± SE (n = 3) for each treatment.

† Average temperature and dissolved oxygen were 17.7 ± 0.1°C (range, 17.0–19.1; n = 69) and 7.6 ± 0.03 mg L^{-1} (range, 6.9–8.4; n = 58).

‡ Data were transformed before statistical analyses.

§ Based on measurements on four occasions, average ammonia concentration was similar (0.002 mg of FAN L^{-1}) in all treatments except the control (0.004 mg of FAN L^{-1}) (FAN, free ammonia-nitrogen, or unionized ammonia-nitrogen).

and particulate nutrient losses over the 2 days. Apparent food consumption (amount of food supplied minus residual food as grams dry weight) was divided by the initial tank biomass, less the initial weights of any mortalities to that point.

Tanks were cleaned, on average, every 6 days. Cleaning involved lowering the water level, siphoning enough water into a 20-L bucket to cover the cages, removing cages to the bucket, draining the tank, scrubbing the tanks and cages, refilling the tanks directly from the preheated adjustment columns, and returning the cages to the tanks, in less than 10 min for any tank. All tank valves were briefly opened each day to remove the organic build-up from the base of the tanks.

Experiment 2: Respirometry at End of Chronic Bioassay

The respirometer system included five elliptical perspex chambers (of 2.31 L) normally set up with two chambers for each treatment and one chamber as control (no animals) (Harris et al. in press). Flow entered each chamber near the base, was continuous, was controlled by a rotameter, and was measured manually twice daily. Flow exiting the top of the chamber was diverted by solenoids to either waste (50 min/h) or to a flow cell containing an Orion oxygen electrode for 10 min/h for data recording. The electrode was automatically calibrated with fully aerated seawater for 10 min in each hour. Values for tanks containing animals were corrected for the oxygen uptake of the control tank, and the final values were divided by the wet weight of animals to provide mg kg^{-1} h^{-1}. The data represented here are average values for the 24 h representing the third (and last) day of each trial.

Commencing Day 64, 29–30 abalone from Treatments 3 (208.64 g) and 2 (195.68 g), respectively, were transferred to respirometer chambers for 3 days (two duplicate chambers for each of two treatments plus one vacant control chamber in each 3-day cycle). Abalone that did not attach to transferable plastic strips were removed manually, either by sliding them directly from the substrate or by inserting a thin, plastic card underneath each abalone's foot. Daily measurements of nitrite concentration, pH, and temperature levels of effluent water from the reservoirs were undertaken, because the chambers were sealed units (Table 3). On Day 69, 30 abalone from two replicates of Treatments 6 (194.20 g) and 5 (233.96 g), respectively, were transferred to the respirometer for the next 3 days, and on Day 73, 30 abalone from two replicates of Treatments 1 (238.42 g) and 4 (197.64 g), respectively, were transferred to the respirometer for the next 3 days.

TABLE 3.

Water quality parameters in respirometer chambers (Experiment 2).

Treatment	NO_2-N (mg L^{-1})*	Temperature (°C)
1†	0.025 ± 0.0002	20.1
2‡	0.52 ± 0.017	20.7
3‡	1.01 ± 0.023	21.9
4†	1.99 ± 0.107	17.1
5§	4.29 ± 0.122	19.0
6§	7.72 ± 0.198	20.5

* Means ± SE, n = 3.
† Flow = 169.1 ± 3.6 mL min^{-1}.
‡ Flow = 164.1 ± 1.7 mL min^{-1}.
§ Flow = 174.0 ± 4.2 mL min^{-1}.

Statistical Analysis

Data were subjected to one-factor analysis of variance after meeting assumptions of normality using the Shapiro-Wilk test (Zar 1996) and homogeneity of variance using Cochran's test (Underwood 1981). Replicates were considered to be independent, and nitrite concentration was analyzed as a fixed factor. Survival data and WWBW:shell length ratio were transformed ($\sqrt{arcsin}$ and log, respectively) before analysis. Results for each nitrite concentration were compared against data for the control (0.024 mg NO_2-N L^{-1}) using Dunnet's test (Sokal and Rohlf 1995). Preliminary analysis indicated that initial size did not affect specific growth rate in this trial. All analyses, including assessment of initial size, survival, unionized ammonia-N, pH, dissolved oxygen, and temperature as covariates (Sokal and Rohlf 1995), were conducted with JMP 3.0 software (SAS Institute).

RESULTS

Experiment 1: Chronic Nitrite Exposure

Specific growth rate (SGR) was significantly affected by nitrite whether SGR was measured on a whole-weight ($p < 0.05$) or shell-length ($p < 0.01$) basis. Growth rates (weight) were, on average, 67.2% of controls (0.024 mg of NO_2-N L^{-1}), regardless of nitrite concentration in the range 0.56–7.80 mg of NO_2-N L^{-1}, although there was considerable variation among replicates (Fig. 1). SGR data for shell length exhibited a similar pattern, with a plateau of growth rates from 61.4 to 54.8% of control values for Treatments 2–5 (0.56–4.15 mg of NO_2-N L^{-1}), except that much more severe growth depression (17.7% of control growth rate) was recorded for the highest concentration (7.80 mg of NO_2-N L^{-1})

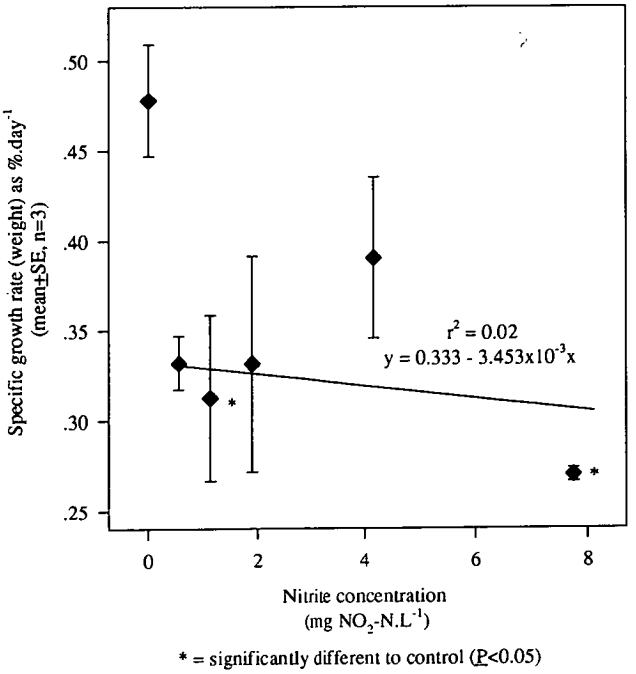

Figure 1. SGR (weight) of juvenile greenlip abalone, *H. laevigata*, subjected to chronic nitrite exposure (mean ± SE, n = 3). The regression line is plotted for 0.56–7.80 mg of NO_2-N L^{-1}.

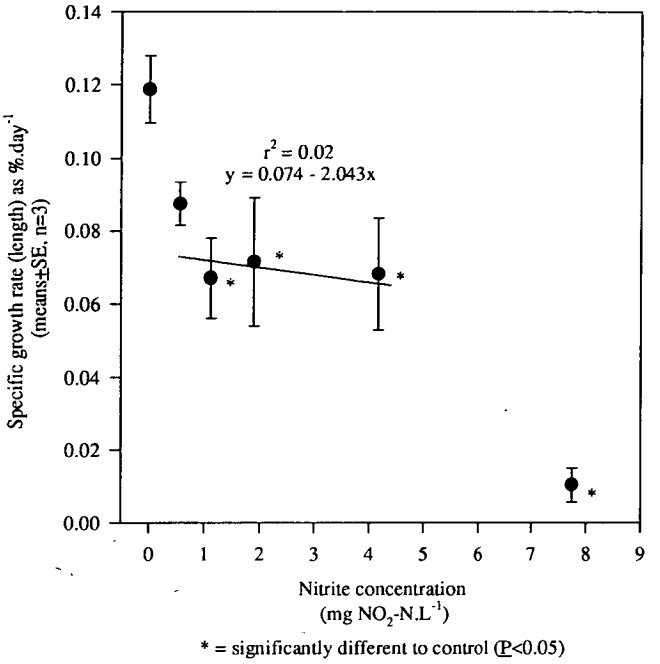

Figure 2. SGR (length) of juvenile greenlip abalone, *H. laevigata*, subjected to chronic nitrite exposure (mean ± SE, n = 3). The regression line is plotted for 0.56–4.15 mg of NO_2-N L^{-1} in order to define the plateau.

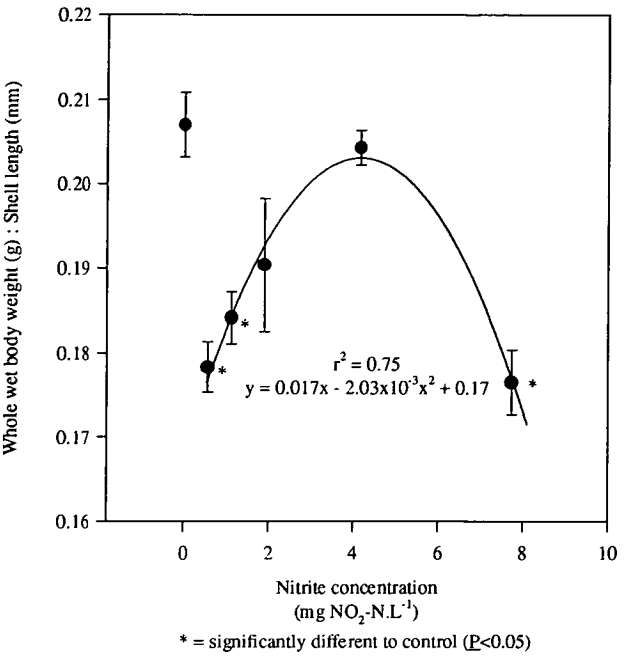

Figure 3. WWBW:shell length of juvenile greenlip abalone, *H. laevigata*, subjected to chronic nitrite exposure (mean ± SE, n = 3).

(Fig. 2). For both sets of data, linear models were fitted to these apparent plateaus.

WWBW:shell length data demonstrated significant differences from the control ($p < 0.01$) at Treatments 2, 3, and 6 (0.56, 1.12, and 7.80 mg of NO_2-N L^{-1}), although no significant differences ($p > 0.05$) were recorded for Treatments 4 and 5 (1.87–4.15 mg of NO_2-N L^{-1}) (Fig. 3). As with the growth data, there were large differences between the control and the remaining treatments, and hence, the quadratic model applied to the data does not include the control.

There was no significant effect of nitrite concentration on food consumption ($p > 0.05$), although it should be noted that the variance was much higher for Treatment 6 (7.80 mg L^{-1}). Survival was not significantly affected ($p > 0.05$) by nitrite concentration (mean ± SE = 82.99 ± 5.07%; n = 6), although the controls were the only treatment with 100% survival.

Experiment 2: Oxygen Consumption Rates at End of Chronic Bioassay

Oxygen consumption rate decreased with increasing nitrite concentration ($p < 0.01$) (Fig. 4). Significant reductions ($p < 0.05$) in oxygen consumption rate occurred in Treatments 5 and 6 (4.29–7.72 mg of NO_2-N L^{-1}) compared with the controls. An exponential decay model was used because of the similarity in oxygen consumption for the two highest concentrations (4.29–7.72 mg of NO_2-N L^{-1}). This model suggests that oxygen consumption will plateau at nitrite concentrations above 7.72 mg of NO_2-N L^{-1}, but without more data, this cannot be presumed.

DISCUSSION

Growth rates of control animals (SGRW = 0.48 ± 0.035% day^{-1}; SGRL = 0.122 ± 0.011% day^{-1}) were comparable with those found in a concurrent trial with greenlip abalone of a similar size, conducted in outdoor ambient tanks (SGRW = 0.305 ± 0.031% day^{-1}; SGRL = 0.107 ± 0.019% day^{-1}) (Maguire et al. 1996). This suggests that the bioassay environment was not directly stressful for the control animals. However, faster growth rates for this species have been recorded in other culture systems at a higher constant temperature (Coote et al. 1996).

Nitrite has been shown to adversely affect growth or food consumption in several aquatic species; however, at least two quite

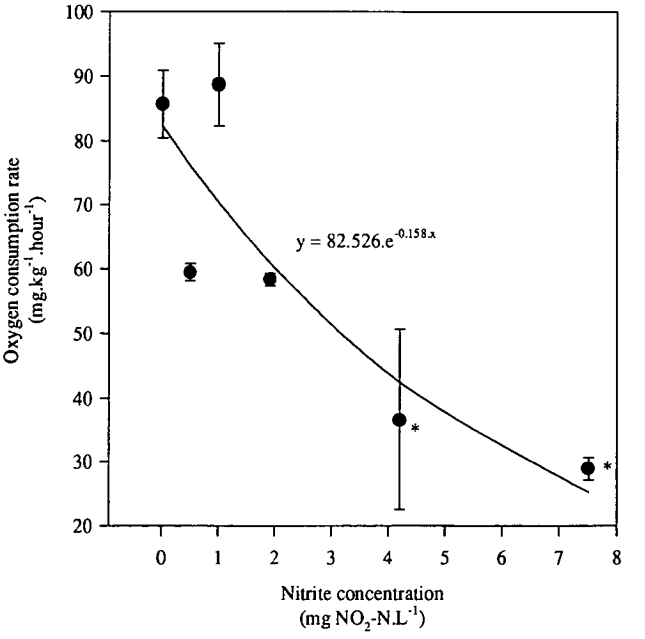

Figure 4. Oxygen consumption of juvenile greenlip abalone, *H. laevigata*, exposed to nitrite (mean ± SE, n = 2).

different dose response patterns have been reported. Wickins (1976) reported a 50% reduction in growth of the marine shrimp *Penaeus indicus* Milne-Edwards at 6.4 mg of NO_2-N L^{-1}, and the growth data (Fig. 5) reflect this trend in whole-weight and shell-growth data for greenlip abalone. As nitrite concentration is increased, growth may be depressed; however, as nitrite concentration is increased further, growth inhibition is not necessarily exacerbated (Figs. 1 and 2). Chen and Chen (1992c) found significant growth reductions for *Penaeus monodon* Fabricius juveniles at and above 4 mg of NO_2-N L^{-1}, again with a plateau being evident in the dose response pattern, particularly for total length data. A similar pattern was obtained by Liu and Avault (1996) for the freshwater crayfish *Procambarus clarkii.* The respiratory pigment in abalone, penaeid shrimp, and freshwater crayfish is hemocyanin. The only study of fish that we can locate on the adverse effects of nitrite on growth is on channel catfish (Colt et al. 1981). In their study, a more typical dose response was obtained, with an initial plateau indicating negligible effect on growth at lower nitrite concentrations, followed by a linear decline in growth at high concentration (Fig. 6). The only available study on molluscs is on the acute toxicity and algal clearance rates of the bivalves, *Crassostrea virginica* Gmelin and *Mercenaria mercenaria* Linné, where levels of 280 mg of NO_2-N L^{-1} caused clearance rate reductions of 89 and 54% for juveniles and 66 and 53% for adults, respectively (Epifanio and Srna 1975).

The apparent decline in oxygen consumption with increasing nitrite-nitrogen concentration may reflect compromised efficiency of the respiratory pigments. The Australian redclaw crayfish, *Cherax quadricarinatus,* demonstrated a similar decrease in oxygen consumption when exposed to nitrite at 100 mg of NO_2-N L^{-1}, although static conditions were used (Meade and Watts 1995). Other studies on carp, *Cyprinus carpio* L., found that when methemoglobin levels rose with exposure to nitrite, arterial oxygen content declined (Jensen et al. 1987, Williams et al. 1992). Similar patterns occur in penaeid shrimp when exposed to nitrite, as pH, oxyhemocyanin, protein, and oxyhemocyanin:protein levels within the hemolymph decline, with the probable result of methemocyanin formation (Chen and Cheng 1995). The hypothesis supplied by Fox (1954), suggesting that many hemoglobin-containing fishes, when swimming quietly, obtain enough oxygen for their needs in the blood plasma, and probably only require an additional supply when they are moving actively, may be useful in this case. In the wild, greenlip abalone exhibit limited movement (mean, 0.5 m mo^{-1}) that tends to increase with declining crevice abundance (Shepherd 1986, Shepherd and Godoy 1989). Greenlip abalone in this study had limited scope for movement in the bioassay cages, and any adverse effects on oxygen loading may have been ameliorated by restricted movement and hence oxygen demand.

In an equivalent study on the effects of ammonia on greenlip abalone (Harris et al. in press), growth results were consistent with food consumption and respiration rate data (as SGRW). As ammonia increased, food consumption was depressed and respiration rate increased, both of which would have contributed to the resultant depressed growth. In this study, nitrite depressed growth and respiration rate but did not affect food consumption. Neither of these trends would necessarily cause depressed growth. It is likely that inefficient use of available energy is occurring; this is consistent with the higher rate of protein catabolism, indicated by ammonia excretion, as observed in penaeid shrimp exposed to nitrite (Chen and Cheng 1995). Clearly, further research in this area is required for juvenile greenlip abalone.

The data for WWBW:shell length suggest that nitrite can affect whole-animal growth (weight) and shell growth (length) differently. We argued that ammonia affected shell growth more that whole-body growth (weight) at low ammonia concentrations, but that this pattern was reversed at high concentrations (Harris et al. in press). The pattern for nitrite is more complex; severe depression of shell growth at the highest concentration (Fig. 2) is not reflected in WWBW:shell length. The low ratio at this concentra-

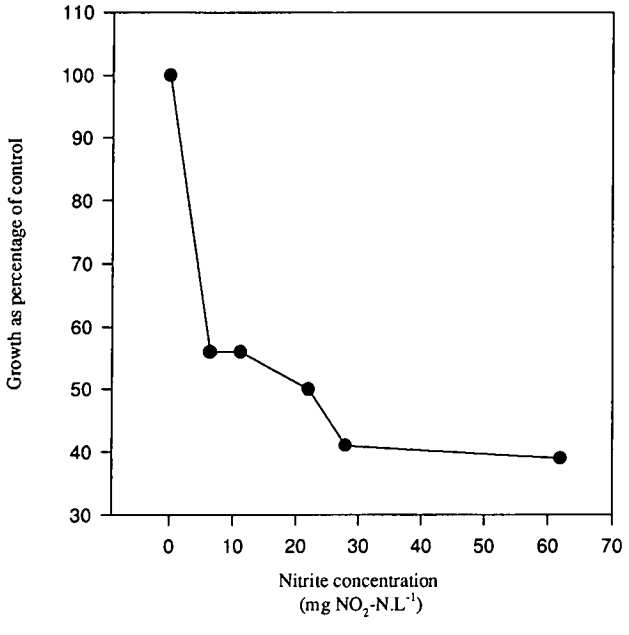

Figure 5. Growth of *Penaeus indicus* subjected to nitrite exposure (after Wickins 1976).

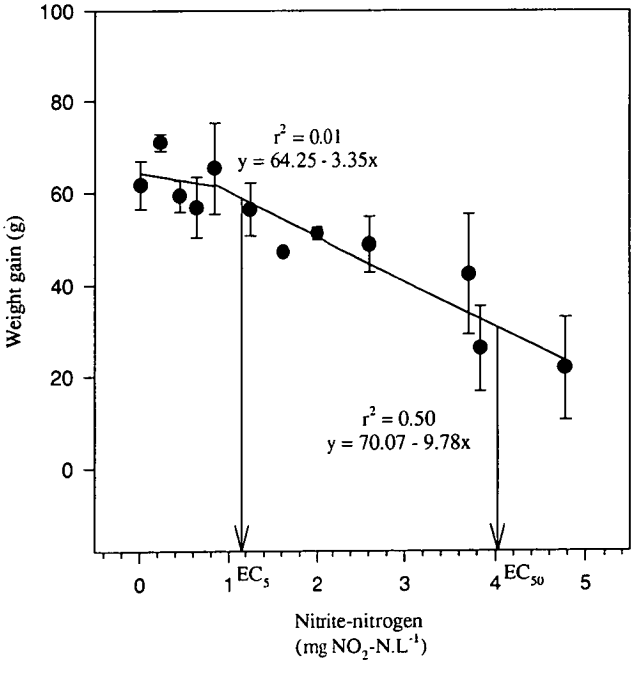

Figure 6. Growth of channel catfish, *Ictalurus punctatus,* subjected to nitrite exposure for 31 days (mean ± SE; n = 3) (after Colt et al. 1981). EC_5 and EC_{50} values were calculated from these data.

TABLE 4.

Growth of various juvenile aquatic animals subjected to nitrite exposure.

Species	Level of NO_2-N (mg L^{-1})	Effect	Author(s)
Penaeus aztecus	4.8	No growth reduction	Wickins 1976
Penaeus merguiensis	4.8	No growth reduction	Wickins, 1976
*Penaeus monodon**	22.45	EC_{50} (weight)	Chen and Chen 1992c
	26.2	EC_{50} (length)	
Penaeus indicus	>6.4	50% growth reduction (weight)	Wickins 1976
Macrobrachium rosenbergii	15.4	Predicted incipient (4 wk) LC_{50}	Wickins 1976
Procambarus clarkii	>2.97	Significant growth reduction	Liu and Avault 1996
Ictalurus punctatus	1.17	Estimated EC_5 (weight)	Colt et al. 1981
	4.01	Estimated EC_{50} (weight)	
Haliotis laevigata	0.56	Growth depression (length and weight)	This study
	1.12	Significant decline in wet weight	

* EC_{50} value was outside of experimental range.

tion may reflect a limitation on whole-body growth imposed by depressed shell growth rates in gastropods (Palmer 1981, Preston et al. 1996). Liu and Avault (1996) reported changes in length gain/weight gain ratio for *Procambarus clarkii* exposed to nitrite.

The results for *H. laevigata* suggest that this species is more sensitive to nitrite than are several other aquatic animal species (Table 4). Similarly, this species is quite sensitive to ammonia (Harris et al. in press), and hence in commercial growout systems, it will be important to minimize nitrogenous wastes. This can be achieved by reduction of dietary protein content (Jirsa et al. 1997), minimization of accumulation of organic matter (through appropriate feed rates, better digestibility, or efficient cleaning systems), or efficient biofiltration in recirculating systems.

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